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# Estrogenic exposure impacts in the frequency and stereological parameters of both induced preneoplastic liver lesions and normal hepatocytes of the brown trout

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*“Caímos, levantamos. Não é opção permanecermos sentados e desenlaçar as mãos, porque o maior poder é acreditar e renascer.”*

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## RESUMO

**Introdução —** O fígado é um órgão vital que desempenha funções fulcrais que mantêm o ser vivo e saudável. O cancro do fígado é a terceira causa mundial mais comum de morte por cancro, sendo a quinta e a sétima forma desta patologia mais frequentemente diagnosticada no homem e na mulher, respetivamente. Em Portugal, a incidência de mortalidade aumentou entre 2006 e 2012, sendo o alcoolismo e a Hepatite B as causas associadas. Os métodos de diagnóstico, tratamento e prevenção do cancro de fígado têm evoluído e se tornado mais eficientes. As neoplasias hepáticas podem ocorrer em toda a filogenia, desde do peixe ao ser humano. O Homem e outros seres vivos estão, permanentemente, expostos a químicos, entre eles, os xenoestrogénios, compostos capazes de replicar a ação dos estrogénios e de interferir em vias celulares, podendo, inclusive, promover a hepatocarcinogénese. No entanto, alguns autores afirmam que os estrogénios podem ter efeito protetor a nível hepático, naquele e noutros processos. Os focos de alterações celulares (FAC) são lesões consideradas pré-neoplásicas, capazes de progredir para tumores benignos ou malignos. Os FAC destacam-se do parênquima normal como agregados hepatócitos com características morfológicas e de coloração atípicas. Neste trabalho experimental, pretendemos avaliar os tipos histológicos de FAC, prevalência e vários parâmetros estereológicos, depois de uma exposição a um estímulo estrogénico, de modo a analisar a capacidade de iniciação/promoção deste tipo de influência.

**Materiais e Métodos —** A indução da hepatocarcinogénese foi realizada em embriões de truta fário, usando o N-metil-N'-nitro-N-nitrosoguanidina (MNNG) como iniciador. Quatro grupos de peixes foram expostos a etanol salino (veículo solvente), 5 µg/L de 17β-estradiol (E2), 50 µg/L de E2 e a 500 µg/L de uma mistura de dois alquilfenóis. Um quinto grupo, não exposto qualquer composto, foi mantido nas mesmas condições. Para a classificação histopatológica de lesões e estimativa da sua prevalência, fragmentos dos fígados extraídos foram fixados e rotineiramente processados para coloração com hematoxilina-eosina. Foram também efetuadas três colorações histoquímicas (ácido periódico Schiff (PAS), azul da Prússia de Perls, e vermelho de Sírius) e conduzidos dois estudos imunohistoquímicos, com anticorpos contra E-caderina e “CYP1A”. Para a avaliação qualitativa, foi implementado um ensaio cego, primeiro recorrendo a microscopia ótica e posteriormente a um sistema de digitalização de lâminas (Olympus VS110); o mesmo foi também utilizado para executar medições e estimar densidades volumétricas ($V_V$). Outros parâmetros estereológicos foram estimados, utilizando um posto de trabalho controlado pelo programa CAST-Grid. Para manuseamento de resultados e análise estatística, foram utilizados os programas Microsoft Excel 2010, VassarStats e STATISTICA 12.

**Resultados —** Os peixes do controlo branco (não iniciados com MNNG) não apresentaram qualquer tipo de lesões hepáticas. Por outro lado, em todos os grupos iniciados com MNNG foram encontrados FAC basofílicos (bFAC), anfofílicos (aFAC) e eosinofílicos (eFAC). Relativamente à prevalência, 100% dos peixes do grupo E2 50 µg/L tinham FAC. O grupo com a menor proporção foi o E2 5 µg/L. Em todos os grupos, o tipo mais frequente de FAC foi o basofílico, à exceção do E2 50 µg/L, e o menos comum o eosinofílico. A média do $V_V$ (bFAC) no fígado atingiu os valores mais altos em todos os grupos — exceto no E2 50 µg/L, onde o $V_V$ (aFAC) foi o mais elevado — revelando que o bFAC atinge a extensão máxima. No que diz respeito ao $V_V$ do somatório dos FAC no fígado [$V_V$ (Total FAC)] e do parênquima total alterado [$V_V$ (PTA)], foi no E2 50 µg/L que se evidenciou quase o triplo do segundo grupo mais elevado. Nos volumes celular e nuclear do hepatócito, comparando o parênquima com e sem lesões, o controlo branco apresentou em todos os parâmetros os valores mais baixos e deferiu dos outros quatro grupos com lesão. No que respeita aos volumes celular e nuclear do hepatócito, no bFAC, o E2 50 µg/L e os alquilfenóis produziram os valores mais elevados, contrariamente ao E2 5 µg/L que promoveu os mais baixos. Observando os resultados do aFAC, a concentração de E2 50 µg/L tendeu a induzir os valores mais elevados e a de E2 5 µg/L os mais baixos. Os FAC foram negativos para as colorações de Perls, e quanto ao vermelho de Sírius verificámos que o estroma perisinusoidal no parênquima normal não diferia em espessura quando comparado com o do FAC. Finalmente, no PAS notámos que, na maioria dos casos, os hepatócitos constituintes dos FAC continham menos teor glicogénico quando comparados com o parênquima adjacente. Quanto à imunoreatividade da E-caderina, verificou-se um sinal negativo em todos os tipos de FAC, quando comparado com o parênquima adjacente. No caso CYP1A registámos um padrão heterógeno.

**Discussão, Conclusão e Perspetivas_—** As lâminas virtuais e a respetiva análise de imagem provaram ser uma ferramenta rápida e precisa em termos de diagnóstico. Ficou claro que a estimulação estrogénica pode agir como promotora quando administrada em altas doses, sendo o E2 ainda mais efectivo, quando comparado com a mistura de alquilfenóis. Analisando os resultados dos alquilfenóis — apesar do potencial estrogénico da mistura usada ser menor do que o do E2 5 µg/L — sugerimos que este possa despoletar outros mecanismos celulares, acabando por originar um maior impacto. Os resultados do E2 5 µg/L apontaram para um potencial efeito protector dos estrogénios, o que vai de encontro ao conceito de hormése (efeitos biológicos diferenciais, derivados da exposição a mais baixos níveis). A classificação dos FAC foi, em alguns casos, dificultada pela heterogeneidade do parênquima e tivemos mesmo a necessidade de introduzir um novo conceito — o Pré-FAC — associado, presumidamente, a estádios iniciais dos FAC. Os nossos dados remetem para a importância do diagnóstico dos subgrupos, sendo a sua classificação e caracterização aspetos essenciais. Os diferentes fenótipos verificados nas

análises imuno- e histoquímicas indicam um cenário morfofuncional múltiplo, que falhou em clarificar a linha evolutiva dos diversos FAC. No que respeita a características de hepatócitos do parênquima normal, as diferenças encontradas entre o controlo branco e os outros grupos indicam que a presença de FAC está associada a alterações no parênquima aparentemente normal. Este facto tem potencial para ser usado como mais um elemento de detecção de processos de carcinogénese, antes da emergência da neoplasia. Estudos futuros sobre este tipo de alterações hepatocelulares e de lesões pré-neoplásicas, nomeadamente a nível molecular, podem ajudar a caracterizar de forma mais detalhada a emergência e progressão dos FAC. Os resultados deste trabalho contribuíram para melhor compreensão das respostas hepáticas à exposição química com iniciadores neoplásicos, seguida de estímulo estrogénico, chamando a atenção para potenciais riscos para as espécies em contacto com estes compostos, incluindo o Homem.

## ABSTRACT

**Introduction —** The liver is a vital organ with a wide range of functions that keep animals alive and healthy. Liver cancer is the third most common cause of death from malignant neoplasms worldwide, being the fifth and seventh most frequently diagnosed cancer in men and women, respectively. In Portugal, the incidence and mortality increased between 2006 and 2012, being alcoholism and infection with hepatitis B associated causes. In parallel, there are growingly efficient approaches for diagnosis, treatment and prevention of liver cancer. Hepatic neoplasms occur across phylogeny, from fish to Man. Humans and wildlife are now continuously exposed to a myriad of chemicals, like xenoestrogens, compounds able to mimic estrogens action and interfere with key cellular processes that lead to hepatocarcinogenesis. Yet, some authors claim that estrogens may have a protector hepatic effect. Foci of cellular alterations (FCA) are preneoplastic lesions capable of progressing, increasing the risk of developing benign or malignant tumors. FCA stand out from the normal parenchyma as aggregates of hepatocytes with abnormal morphology and staining features. In this experimental work we detected and evaluated FCA histological types, prevalence and several stereological parameters, after exposure to estrogenic stimuli, in order to analyze the initiation/promotion capacity of such type of influence.

**Materials and Methods —** Hepatocarcinogenesis induction was made in brown trout embryos, using N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as the initiator agent. Four fish groups were exposed to ethanol saline (dissolving vehicle), 5 µg/L of 17β-estradiol (E2), 50 µg/L of E2 and 500 µg/L of a mixture of two alkylphenols. For histopathological classification of the lesions and estimation of their prevalence, livers fragments were fixed and routinely processed for hematoxylin–eosin staining. Also, three histochemistry stainings were made (period acid-Schiff (PAS), Perls' Prussian blue and Sirius red) and two immunostaining studies were conducted, with antibodies against E-cadherin and CYP1A. For qualitative assessment, a blind assay was implemented, first resourcing to optical microscopy and then to an Olympus VS110 virtual slide scanning system; also used for area measurements to estimate volume densities. Other stereological parameters were estimated using a workstation controlled by the software CAST-Grid. For data handling and statistical analysis, we used Microsoft Excel 2013, VassarStats and STATISTICA 12.

**Results —** Fish from the blank (non-MNNG initiated) control did not have any sort of liver lesions. By the contrary, basophilic, amphophilic and eosinophilic FCA were found in all the MNNG initiated groups. Regarding the prevalence, the E2 50 µg/L group reached a remarkable result, with 100% of fish having FCA, and the group with the lowest % value was the E2 5 µg/L. In all groups, the most frequent FCA type was the basophilic, except in the E2 50 µg/L, and the less common the eosinophilic. The mean $V_V$ (bFCA) in the liver

reached the highest values in all groups — except in the E2 50 µg/L group, where the $V_V$ (aFCA) was greater — revealing that the bFCA attained maximum spreading. Concerning the $V_V$ of all FCA together in the liver [$V_V$ (Total FCA)], and that of the total altered parenchyma [$V_V$ (TAP)], the E2 50 µg/L group reached almost the triple of the second highest group. As to the cell and nuclear volumes of the hepatocyte, comparing parenchyma with and without lesions, the Blank Control group presented in all the parameters the lowest values and differed from the other four groups with lesions. Additionally, regarding the cell and nuclear volumes of the hepatocyte within the bFCA, E2 50 µg/L and Alkylphenols achieved the highest values, contrary to E2 5 µg/L, that presented the lowest. Observing the results from aFCA, E2 50 µg/L tends to promote the higher values and E2 5 µg/L the lowest. The FCA were negative for Perls', and the Sirius red showed the delicate perisinusoidal stroma did not differ in thickness in the FCA compared with non-lesioned areas. PAS showed that in most cases there was less glycogen within the FCA hepatocytes, when compared with the close parenchyma. Finally, we saw a negative pattern regarding E-cadherin immunoreactivity, within all FCA types, when compared to the normal parenchyma, while for CYP1A we verified heterogeneity in the immunostaining patterns.

**Discussion, Conclusion and Perspectives —** The virtual slides and related image analysis proved to be valuable for a faster and accurate diagnosis. It was clear that estrogenic stimulation can act as a promoter when administrated in high doses, being E2 even more effective compared with the alkylphenols mixtures. Observing the results of Alkylphenols, despite its lowest estrogenic potential compared with E2 5 µg/L, we considered that it may trigger other cellular mechanisms besides estrogenicity, ending up causing more impacts. The E2 5 µg/L outcomes pointed for a potential protector effect of estrogens, which is in line with the concept of hormesis (differential low dose-response phenomena). The classification of FCA was, in some cases, hampered by the parenchyma heterogeneity and we even felt the need to introduce a new concept — Pre-FCA — associated to the presumably earliest stages of FCA. Data backs the importance of sub-groups diagnosis, being the FCA classification and characterization an essential aspect. The different phenotypes verified in the histochemical and immunochemistry analyses pointed to a multiple morpho-functional scenario, that failed in clarify the evolution line of the diverse FCA. The differences between hepatocytes from blank control and the other groups, regarding the parenchyma comparison, indicated that changes can occur in the apparently normal parenchyma, adjacent to FCA. This fact has potential to be used as one early signal that neoplastic lesions may be emerging or do already exist. Future studies of these kinds of early presumptive preneoplastic changes and also lesions, especially at a molecular level, should help to characterize with more detail the FCA. Overall, the findings of this work contributed to enrich the understanding of liver responses after exposure to a neoplastic initiator chemical agent followed by chronic estrogenic stimulation, and draw attention to potential risks for species in contact with these compounds, including Humans.

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# INTRODUCTION

# INTRODUCTION

## LIVER CANCER

### Epidemiologic background

The liver is a remarkable organ with major relevance in maintaining homeostasis and proper functioning of the whole organism, and therefore it has been a study target to scientists, not for decades, but actually for centuries (Bryan, 1930; Loukas et al, 2011). Among its main functions, we can describe: the metabolism of lipids, carbohydrates and proteins; storage of glycogen, vitamins and iron; detoxification of noxious chemicals and bile production. In the liver, the nutrients absorbed in the digestive tract are processed and stored to be used by other organs, functioning as an interface between the digestive system and the blood. When the liver is injured, several extra hepatic organs may be affected secondarily — and even compromised — by alterations in the levels of metabolites levels that it regulates (Arias et al, 2009).

In economically developed countries, cancer is the leading cause of death and the second one in developing countries. The incidence of cancer is increasing in economically developing countries as a result of population aging and growth as well as, gradually, due to an adoption of cancer-associated lifestyle choices including smoking, drinking, physical inactivity, and 'westernized' diets (which include excessive red meat, sugary desserts, high-fat foods, and refined grains) (Jemal et al., 2011). In men, liver cancer is the fifth most frequently diagnosed cancer worldwide (523,000 cases/y, 7.9% of all cancers), being the second most frequent cause of death by cancer. In women, it is the seventh most commonly diagnosed cancer (226,000 cases/y, 6.5% of all cancers) and the sixth leading cause of cancer death. Liver cancer has a high mortality rate; the geographic distribution of mortality is similar to that of incidence. An estimated 748,300 new liver cancer cases and 696,000 cancer deaths occurred worldwide in 2008 (478,000 in men and 218,000 in women), being half of these cases and deaths estimated to occur in China. Because of its high fatality (overall ratio of mortality to incidence of 0.93), liver cancer is the third most common cause of death from cancer worldwide (Ferlay et al., 2010; Jemal et al., 2011).

The most common histological type of liver malignant neoplasm is hepatocellular carcinoma (HCC), accounting from 70% to 85% of the total liver cancer burden worldwide. Other forms include: (i) childhood hepatoblastoma, and (ii) adult cholangiocarcinoma (originating from the intrahepatic biliary ducts) and (iii) angiosarcoma (from the intrahepatic blood vessels) (Chuang et al., 2009; Jemal et al., 2011; Serag, 2012). HCC carcinogenesis is a complex process that involves several modifications in the molecular path-

ways and genetic alterations, which ultimately lead to malignant transformation and disease progression (Sanyal et al., 2010).

About 80% of HCC cases occur in Sub-Saharan Africa and in Eastern Asia, with typical incidence rates of more than 20 per 100,000 individuals (Serag, 2012). This disturbing scenario is most likely due to: 1) failure to recognize and follow the high-risk individuals; 2) high prevalence of specific risk factors in the populations; 3) lack of medical expertise and facilities for early diagnosis; and 4) the absence of an effective treatment after diagnosis. Other important factors include: 5) poor compliance, with inadequate attendance or absent in surveillance programs and thus late discovery of patients with already severe tumor status; 6) low awareness of the benefits of HCC treatment and prevention actions; and 7) screening depreciation by some physicians (Ferenci et al., 2010). Southern European countries (such as Spain, Italy, and Greece) tend to have mid-incidence levels (10.0 to 20.0 per 100,000 individuals), whereas North America, South America, Northern Europe, and Oceania have a low incidence of HCC (<5.0 per 100,000 individuals). Recent decreases in the incidence of HCC were reported in high-incidence areas, like among Chinese populations living in Hong Kong, Shanghai, and Singapore; the incidence in Japan also is decreasing. This is probably due to the hepatitis B virus (HBV) vaccine, since, as of 2008, a total of 177 countries (91%) had introduced the HBV vaccine into their national infant immunization schedules. In 2006, only 27% of infants worldwide received the first dose within 24 hours post birth, as recommended by the World Health Organization (WHO). On the other hand, cases of HCC are increasing in low-incidence areas such as the United States, Canada and Central Europe, possibly due to the obesity epidemic and the rise in hepatitis C virus (HCV) infection through continued transmission by injection drug users (Jemal et al., 2011; Serag, 2012).

As stated by Serag (2012), HCC is not frequently detected at the first 4 decades of life, except in populations characterized by a hyperendemic HBV infection. The mean ages of diagnosis with HCC were 55–59 years in China and 63–65 years in Europe and North America. In low-risk populations, the highest incidence of HCC is among individuals aged 75 or older. HCC is predominant among men, with the highest male:female ratios found in areas of high incidence (overall sex ratio male:female is 2.4) (Ferlay et al, 2010).

## Portuguese context

Unfortunately, we have been witnessing the beginning of alcohol consumption at very early ages, the generalization of overconsumption in girls and the frequent adoption of "binge drinking" (drunkenness). Alcohol is related with several diseases, however, in Portugal, it has been having a high impact in alcoholic cirrhosis and deaths from road ac-

cidents, particularly in young people (Marinho, 2008). In the top of the 20 countries with highest beverage-specific adult per capita consumption, Portugal occupies the third place regarding the wine consumption, being also considered the wine producers with a high level of production (World Health Organization, 2004). The alcohol consumption in Portugal is 15% higher than in the rest of Europe and it is estimated that there are approximately 1 million alcoholics or excessive alcohol drinkers in Portuguese population (Mota et al, 2010). In Portugal, adult per capita consumption of alcohol is mainly characterized by consumption of wine, followed by beer and the total adult per capita consumption of pure alcohol in Portugal rounds the 14.6 liters. Concerning the mortality rates, from death causes where alcohol is one of the underlying risk factors, liver cirrhosis clearly stands out (World Health Organization, 2004 and 2010). On the other hand, the hepatic cirrhosis, one of the most serious consequences of alcoholism, was estimated to be the tenth leading cause of death and about two-thirds of the cirrhosis cases were rated as of alcoholic etiology. In line with the cirrhosis, in the same year, 329 Portuguese died with hepatocellular carcinoma (Marinho, 2008). In Portugal the legal age for alcohol consumption is 16 years, but consume before age 18 increases the risk of addiction and studies show that 40% of young people who start drinking excessively at age 13 are alcohol dependent and in Portugal people start drinking too early, exactly at an average age 13 (Marinho, 2008).

Another serious situation that calls urgent attention is the phenomena occurring in Portugal and in Europe called the “feminization of alcoholism”, namely because girls, at least as to spirits, are drinking (at least) as much as boys. This sudden increase in alcoholic liver disease among women showed their susceptibility to the hepatotoxic effects of alcohol. Women tended to present more severe liver disease than men, particularly alcoholic hepatitis, after a shorter period of excessive drinking and at a lower daily alcohol intake. Differences in body size and composition (% of water, for example) may be partly responsible for the greater susceptibility of women, but differences in immune reactivity between the sexes may also play a part (Mota et al., 2010; Marinho, 2008).

Besides alcoholism, the infection with hepatitis B virus (HBV) is also a public health problem in Portugal, despite its prevalence is relatively low: approximately 1%. However, a national serological survey in 2004 pointed to 0.36% individuals chronically infected with HBV (Mota et al., 2010). In Portugal, the second leading cause of hepatic disease is viral hepatitis. The mortality from liver cirrhosis has approximately 2,500 cases per year and according to statistics of the hospitals, 15% to 20% of patients with liver cirrhosis are infected with HBV (Mota et al., 2010). Fortunately, the vaccination is obligatory for all newborns in Portugal since 2000 and the 2nd and 3rd doses are given at 2 and 6 months of age respectively (National Vaccination Program since January 1, 2012)

Using the National Cancer Register as a database of all malignant tumors in the population residing in Portugal, in 2006, we can verify: an annual incidence of 3.94, with the registration of 417 cases (1.1%) — 316 males and 101 females, and an annual mortality of 6.51, with a record of 689 deaths (3.2%) — 491 males and 198 females (Registo Oncológico Nacional 2006, 2012). Consulting the datasheet of GLOBOCAN, we can see that the estimated liver cancer incidence in Portugal, regarding the year of 2012, is 2.7 (772 cases) in men against 16.1 (293318 cases) in China; in women is 1.1 (232 cases) against 8.2 (101452) in China. The estimated liver cancer mortality in men is 4.6 (655 deaths) against 19.7 (281802 cases) in China and in women is 2.6 (253 deaths) against 13.1 (101401 deaths) in China. Comparing the result sets of the two years, we can conclude that the number of cases and mortality increased, supporting the need of new prevention and early diagnosis approaches.

The most recent epidemiological data on cancer in Portugal is the 2011 Oncologic Registry of the Portuguese Oncology Institute Porto (Registo Oncológico 2011, 2013), where we can observe that of the 53 malignant liver tumors registered (0.75%), 36 were of male and 17 were of female patients and that the age groups more affected were 55-59 and 65-69. The number of metastatic tumors (16 in 53) is the highest value, suggesting late diagnosis, which decreases efficacy of the treatment and the chance of patient survival (Hemming et al, 2001; Llovet and Bruix, 2003). With concern to the histological types, 7 were clinically diagnosed as malignant, 14 as cholangiocarcinoma, 29 as hepatocellular carcinoma (this being the most frequent type, in line to what is verified worldwide), and 3 as other tumors.

## Etiology and risk factors

There are multiple etiologic factors involved in liver pathological process, leading to HCC, and all of them depend on the geographic location, which has a direct impact on the characteristics of the patients, influencing the disease course and therefore making HCC an extremely complex condition associated with a poor prognosis (Sanyal et al., 2010).

Chronic infection with hepatitis B virus (HBV), hepatitis C virus (HCV) or both is the most common cause of HCC worldwide. HBV and HCV promote cirrhosis, which is found in 80%–90% of patients with HCC (Serag, 2012). These two viruses account for 78% (HBV, 53%; HCV, 25%) of the total liver cancer deaths globally, with the estimates by region varying from about 64% in the Americas to about 90% in Japan and Singapore (Jemal et al., 2010). Approximately 5% of the world population (350 to 400 million people) is chronically infected with HBV; 75% of infected people are Asian, with a lower prevalence (0.3%– 1.5%) in Western countries (Serag, 2012).

HBV is a partially double-stranded DNA-containing virus belonging to the Hepadnaviridae family, and its infection can induce HCC through both direct and indirect pathways (Sanyal et al., 2010). HBV infection starts by inducing hepatocyte injury and chronic necroinflammation, with subsequent hepatocyte proliferation, fibrosis, and cirrhosis. The continuous regeneration in cirrhosis causes an increase in the liver cell turnover and an accumulation of mutations in the host genome that can result in genetic alterations, chromosomal rearrangements, activation of oncogenes, and inactivation of tumor suppressor genes. However, HBV can also leads to HCC in the absence of cirrhosis through a direct oncogenic effect, since it is able to integrate its DNA into host cells and act as a mutagenic agent, causing secondary chromosomal rearrangement and increasing genomic instability (Azam and Koulaouzidis, 2008; Sanyal et al., 2010).

HCV infection causes chronic inflammation, cell death, proliferation, but when related to HCC is found almost exclusively in patients with cirrhosis. Thus, the risk for developing HCC is higher in HCV-infected patients, and it depends on the degree of liver fibrosis at the time of HCV infection (Sanyal et al., 2010). HCV belongs to the *Hepacivirus* genus of the Flaviviridae family. Until now, there is no evidence to support that HCV integrates into the cellular genome or has another direct role in the molecular pathogenesis of HCC. On the other hand, HCC can develop via HCV induced chronic liver injury, progressing to fibrosis and cirrhosis (Hamilton et al., 2000; Sanyal et al., 2010).

Alcohol-induced liver injury is a leading cause of liver cirrhosis among Western populations, being the most important HCC risk. The excessive alcohol consumption increases the risk of HCC, primarily, through the development of cirrhosis. It has been suggested that heavy alcohol consumption of > 80 g/d ethanol for at least 5 years increases the risk of HCC by nearly fivefold. The risk seems to be proportional to the amount of alcohol consumed. In addition to a daily dose response, persistent consumption can cause long-term effect on the risk of HCC occurrence. An association between genetic polymorphisms of the enzymes participating in the metabolic pathway of ethanol and the increased risk of HCC in heavy alcohol drinkers has been also proposed as a mechanism leading to HCC (Blonski et al., 2010). In addition, Chuang et al (2009) suggested that HCV infection can accelerate the progress of alcoholic liver disease and lead to the development of HCC at a younger age among drinkers than among non-drinkers. Therefore, the authors concluded that alcoholic cirrhosis may assume a greater relevance as one of the risk factors for HCC in populations with low prevalence of HBV and HCV infection and low exposure to aflatoxins, such as North America and Northern Europe.

Based on the stated above, there is no doubt that cirrhosis increases significantly the risk of HCC development. Cirrhosis develops following long periods of chronic liver disease and is characterized by a decrease in hepatocyte proliferation, which points to an

exhaustion of the regenerative capacity of the liver. During this pathological process, we can verify an increase in fibrous tissue and a destruction of liver cells, providing the necessary background for development of cancerous nodules (Sanyal et al., 2010). Therefore, the persistent infection of HCV and chronic alcoholism leads to the development of cirrhosis due to perpetual and impaired wound-healing process and during which, the repeated repair and regeneration process, can cause genomic aberrations and mutations, promoters of carcinogenesis. We must be aware that in old individuals this can be even more problematic, since, due to the aging, the ability of precise DNA repair is depleted, contributing to cellular aberration (Tsuchishima et al., 2013). Liver cirrhosis can have a significant impact on liver reserve and is often an integral part of the morbidity and mortality associated with HCC, reason why the presence and severity of cirrhosis must be diagnosed in all patients in order to assess prognosis and decide the appropriated treatment measures (Sanyal et al., 2010).

Iron is also relevant, as its altered metabolism seen in hereditary hemochromatosis (HH) is responsible for the excess storage of that metal in the liver and the subsequent development of liver dysfunction. According to Blonski et al. (2010), several population-based and case-control studies have demonstrated that HH clearly confers an elevated risk for the development of HCC. However, the effect of iron excess appears to be independent from development of cirrhosis and may interact with alcohol and HBV/HCV infections (Chuang et al., 2009).

In Southern China and sub- Saharan Africa, dietary ingestion of high levels of aflatoxin may present a special environmental hazard, particularly in persons chronically infected with HBV (Xia et al, 2013). Aflatoxin B1 (AFB1) is a mycotoxin produced by fungi of the genus *Aspergillus* (*Aspergillus flavus* and *Aspergillus parasiticus*) that grows readily on cereals, peanuts and other vegetables stored under conditions of high temperature and humidity. In animals, AFB1 is a proven powerful hepatocarcinogen, leading the International Agency for Research on Cancer to classify it as a carcinogen. Once ingested, AFB1 is metabolized to an active intermediate, which binds to DNA and cause genetic and epigenetic alterations, including the mutation of the *p53 and p16* tumor suppressor genes and DNA hypermethylation. The carcinogenic role of aflatoxins, in particular of AFB1, has therefore been confirmed and shown to be independent from — and to interact with — that exerted by HBV infection (Chuang et al., 2009; Blonski et al., 2010; Sanyal et al., 2010; Serag, 2012).

Other factors have also been incriminated in HCC, including dietary factors, obesity, diabetes and long-term use of oral contraceptives, as we can verify in the further examples. As to diet, there is evidence supporting that consumption of yogurt and milk as well as vitamin supplements offers a protective effect against HCC (Blonski et al., 2010).

Several data also have been reported on a potentially favorable effect of coffee on liver function and liver diseases, including liver cancer (Chuang et al., 2009), by reducing the risk of cirrhosis, however the involved mechanisms still unclear. Coffee drinking also might protect against HCC by reducing levels of insulin and thereby the risk for type 2 diabetes, a risk factor for fatty liver disease, cirrhosis, and HCC (Serag, 2012).

Diabetes, a condition closely associated with obesity (nowadays recognized as a significant risk for the development of several types of cancers), has been proposed as a risk factor for both chronic liver disease and HCC. The potential mechanism from obesity and diabetes to HCC may be through fatty liver or non-alcoholic fatty liver disease (NAFLD) (Chuang et al., 2009). Sanyal et al (2010) described that NAFLD occurs in the absence of alcohol use, although the hepatic histology seems to be similar to the one observed in alcoholic hepatitis, with histological alterations including hepatic steatosis, inflammation, hepatocyte injury and fibrosis. NAFLD pathological process comprises a different conditions, at first with the presence of fat alone, then plus inflammation, fat plus ballooning degeneration, and non-alcoholic steatohepatitis (NASH), the latter being the most serious form of NAFLD. This ultimate stage, through a process of chronic inflammation and subsequent hepatic fibrosis, can lead to cirrhosis, which itself is an independent risk factor for the development of HCC (Sanyal et al., 2010). Despite all this, the impact of NASH on the incidence of HCC may well be underestimated (Blonski et al., 2010).

The use of combined estrogen–progestogen oral contraceptives (OCs) significantly increases the risk of liver adenomas, and is associated with the risk of HCC, although the absolute risk is likely to be small and has been shown in populations at low HBV risk (Chuang et al., 2009). However, as described by Blonski et al. (2010), benign liver tumors were hardly observed in young women before the widespread use of oral contraceptives. He also points to the fact that OCs therapy can to be associated with the development of benign liver tumors (hepatic hemangioma, hepatocellular adenoma or focal nodular hyperplasia) and even of malignant liver tumors, hypothesis that needs to be confirm with further research. The same author states that it is not frequent malignant transformation occur within the context of hepatic adenomas and that it is unclear whether the use of OCs influences the possibility of developing adenoma its malign transformation. To evaluate further the risk of HCC in the setting of OCs use, several observational studies have been conducted, however, based on its results, it was not possible to achieve a final conclusion regarding the association between OCs, the risk of HCC and how such risk is modified by duration of OCs use (Maheshwari et al, 2007).

Finally, there is a risk of developing liver cancer by exposures to chemical agents, especially in occupational settings, with both hepatocellular carcinoma and angiosarcoma

being associated with diverse “culprit compounds”, even if epidemiological evidence is not always clear (Bosetti et al., 2003; Uccello et al., 2012); this aspect is further explored below.

## Diagnosis

Long-term survival requires the detection of small tumors, often present in asymptomatic individuals, which may be not willing to invasive therapeutic options. Surveillance of high-risk individuals for HCC is commonly performed using the serum marker alfafetoprotein (AFP) often in combination with ultrasonography. Diagnosis of HCC often requires more sophisticated imaging modalities such as computerized tomography (CT) scan and magnetic resonance imaging (MRI) (Bialecki and Bisceglie, 2005). However, it is sufficient to establish a diagnosis of HCC combining the finding of: the classic appearance on one of the imaging modalities (large and/or multifocal hepatic mass with arterial hypervascularity); and elevated serum AFP, against a background of chronic (generally asymptomatic), generally cirrhotic-stage liver disease. The standardization of diagnosing HCC by non-invasive imaging has allowed earlier HCC detection and has facilitated the implementation of surveillance programs (Cabrera and Nelson, 2010; Ferenci et al., 2010).

The definitive diagnosis of HCC depends on histological examination of lesions, especially in AFP-negative patients. Ultrasound- or CT-guided percutaneous biopsy offers sufficient tissue for histological diagnosis and the risk of bleeding or seeding of tumor cells along the needle tract is minimal. In patients with significantly elevated AFP levels who are potentially eligible for HCC resection or liver transplantation, liver biopsy is not suggested due to the risk of tumor cells spreading before surgery (Hamilton and Aaltonen, 2000).

## Treatment

According to Ferenci et al. (2010), treatment approaches depend on the disease stage at diagnosis (liver function, tumor size, and the presence or absence of metastatic lesions or vascular invasion) and on access to complex treatment regimens. However, most of the treatment options are expensive and/or require specialized centers and, unfortunately, advanced disease is not curable, being its management expensive and only marginally effective in increasing quality-adjusted life years. In most cases, curative treatments are not feasible, limiting the options to palliation. The screening of high-risk populations is the only way of detecting tumors at a stage at which they are capable of being treated.

There are several potentially curative treatment modalities available for patients with early stage HCC, including: surgical resection (patients without advanced cirrhosis and with well-preserved liver function and normal portal pressure), liver transplantation (HCC patients with decompensated cirrhosis), and local ablation (patients who are not candidates for surgical treatment) (Kanwal et al., 2012).

The tumor biology of HCC and the co-existing cirrhosis make drug development more difficult. Fortunately, over the past years, significant progress was made in the elucidation of HCC molecular pathogenesis, which led to the development of molecular-targeted agents such as sorafenib (Cabrera and Nelson, 2010). As chemotherapy can reactivate the virus in HBV carriers, patients with HBV or HCV require antiviral therapy to control viral replication and improve cirrhosis-related outcomes (Sanyal et al., 2010).

## Prevention

As stated by Jemal et al. (2011), “A significant proportion of the worldwide burden of cancer could be prevented through the application of existing cancer control knowledge, and by implementing programs for tobacco control, vaccination (for liver and cervical cancers), and early detection and treatment, as well as public health campaigns promoting physical activity and healthier dietary patterns”. From this basis we can think of numerous ways to avoid liver cancer, at least for most known etiologies. For instance, one effective strategy for reducing the risk of HCC is the vaccination against viral hepatitis (HBV). In contrast to HBV, no vaccine is available against HCV. Therefore, its prevention measures include screening of blood, organ, tissue, and semen donors for antibodies to HCV and implementing proper infection control practices during all medical, surgical, and dental procedures (Ferenci et al., 2010; Jemal et al., 2010). Another relevant prevention method and probably undervalued, is the health education about viral hepatitis, where should be emphasizing the ways in which it is possible for the disease to spread in relation to, as described/grouped by Ferenci et al. (2010): local practices involving blood-blood contact (circumcision, scarification, tribal marks, and tattoos); care of open sores and marks after multiple-use tooth extraction equipment; and reuse of needles.

In the cases where the vaccination is not available, prevention procedures include, among other, screening of donor’s blood for antibodies to HCV and instituting adequate infection control practices during medical procedures (Jemal et al., 2011). It is also essential to implement sanitary conditions and reduce the contamination of food with aflatoxins through adequate food storage system (Chuang et al., 2009). Control of alcohol drinking and tobacco smoking, adoption of a balanced diet and a not sedentary life to decrease the levels of obesity, represent others factors to consider (Chuang et al., 2009).

It cannot be also forgotten that screening should be encouraged, mainly in regions in which it is possible to offer curative treatment for HCC. The main risk factors for HCC are well known, and this allows cost-effective surveillance, being the screening for early detection of HCC recommended for the groups of high-risk patients. Surveillance must involve the establishment of screening tests, screening intervals, diagnostic criteria, and recall procedures (Ferenci et al., 2010).

## ENVIRONMENT AND CHEMICAL CARCINOGENESIS

Despite the considerable efforts to decrease environmental pollution, one of the most serious "worldwide sicknesses" caused by humankind, what we still witness every day is the continuous and often uncontrolled introduction of new compounds in living and working environments. The complexity of these problems is huge, but as Fucic et al. (2012) stated: "The balance between needs of a fast growing human population and technology/science development is questionable, partially as a consequence that the available knowledge is not always applied in an efficient way as it should be".

In the last century, numerous agents have been associated as causes of several diseases, including cancer, which allow researchers to identify pathological pathways and point new ways of prevention and treatment. Regarding the pollution related agents, the solution clearly passes through reducing the pollution and preventing exposure. However, despite all the scientific advances, including the significant information collected due to the development of molecular biology techniques, several fundamental questions about the environmental threats to human health are still without answers (Fucic et al., 2012).

Thus, people (and a vast range of animals, including vertebrates) are continuously exposed exogenously to varying amounts of chemicals that have been shown to have carcinogenic or mutagenic properties, reason why over the last decades, scientists have been studying the impact that these noxious and toxic substances have on human (and animal) health. Humans are never exposed to only one chemical, by the contrary, every time they come into contact with a myriad of compounds, which may be absorbed into our bodies and are never fully eliminated (Yardley, 2004). All of this is caused by our industrialized society that has an excessive abundance of toxic chemicals being used in daily basis in food and environment, everything into an attempt to control nature, for instance with uncountable pesticides and herbicides or overuse of lasting food. In addition, safety analysis/toxicity tests conducted on these chemicals are typically done on one single chemical at a time and do not investigate the cumulative impact numerous chemicals have in our bodies over many years (Yardley, 2004; Wogan et al., 2004).

The influence of the environment can be seen in the differences in cancer rates throughout the world and the change in cancer rates when groups of people move from one country to another, indicating that different environmental exposures are linked to specific types of cancer. As previously stressed, the good news is that a large number of cancers can be prevented by avoiding risk factors (National Cancer Institute, 2003).

Regarding the chemical carcinogenesis, and according to Bailey et al., 1987; Rotchell et al, 2008; Vincent and Gatenby, 2008; Irigaray and Belpomme, 2010) there is an inherent progress that comprises three sequential and successive steps across phylogeny — initiation, promotion and progression — being the carcinogens able of assume different roles:

- Initiators can be defined as carcinogens capable to induce a first “driver” mutation in a dividing cell, through direct or indirect mutagenesis, leading to emergence of a primary clone of mutated cells with a permanent genomic damage. Therefore, initiation is an irreversible mutation in the DNA of a somatic cell, inducible by experimentation that confers a permanent increase in susceptibility to cancer formation. Carcinogen exposure regimes given to fish, for instance, often involve larval and juvenile stages, which are characterized by rapid growth and sensitivity to such compounds, probably due to very fast cell division in growing tissues. The morphologic indicator of initiation in liver is the formation of the so-called foci of cellular alteration (FCA), as will be approached further on. Although necessary, initiation, the first carcinogenesis step or stage, is not a sufficient condition for the development of the tumor;
- Tumor promoters can be classified as non-genotoxic carcinogens that typically do not directly affect DNA and have an effect that is actually reversible, depending on the exposure interval. They are capable of causing clonal expansion of initiated cells, by inducing proliferation of mutated cells, preventing these from apoptotic loss and providing the acquisition and preservation of additional genetic and/or epigenetic changes. In the context of liver carcinogenesis, the initiated cells (focal clones) show preferential growth when compared to nonfocal tissue nearby. In promotion, the so-called reversible stage of neoplasia, cell proliferation is required for clonal expansion of initiated cells;
- Finally, tumor progressors can be considered carcinogens that advance mutated cells from promotion to progression, allowing premalignant mutated cells to irreversibly acquire the phenotype of fully malignant cells. Progression can involve the accumulation of further genetic alterations in a population of initiated cells that have been provided a growth advantage through promotion. Tumor cell heterogeneity may serve as evidence of progression, which can result from genetic instability acquired during tumor progression. If genetic instability is ongoing, these cells are mutated at rates in excess of those of the surrounding tissue producing subclones — and some of these would be ex-

pected to have adaptations that give them a selective advantage. For instance, they could develop mechanisms to avoid the host's immune defense system and/or obtain increased invasive capacity, thus enabling the tumor to extend, invade and metastasize.

## Animal models in (chemical) carcinogenesis – From mouse to trout

Biomedical research depends on the use of both animal testing and specific animal models to understand the pathogenesis of human diseases at a cellular and molecular level and to provide systems for developing and testing new therapies and chemicals. For many years, animals with cancer have been used as model systems for studying a wide variety of neoplasms that also occur in human beings. Animals with spontaneously-developing cancers that are prevalent also in humans, such as lymphoma, bladder cancer, and melanoma, are vital resources that facilitate investigations regarding the diseases pathogenesis and response to treatment (LeRoy, 2013; Lieschke et al., 2007).

Mammalian models, like the mouse, have been pre-eminent in comprehending human diseases, mainly because of the striking homology between mammalian genomes and the many similarities observed from anatomy to cell biology and physiology. Advanced transgenic approaches using dominantly acting disease-causing transgenes have allowed the creation of mouse models that accurately replicate the pathology of human diseases, being the generation of cancer models through the tissue-specific expression of oncogenes a noteworthy example. Furthermore, the development of specific allelic modifications through gene targeting by homologous recombination has become the mouse the most broadly used model of human disease (Lieschke et al., 2007).

The history of chemical carcinogenesis is characterized by key epidemiologic observations, but where animal experiments have also an essential role in the identification of cancer-causing chemicals. However, after the verification of carcinogenesis at the cellular level as irreversible process, the target is now the mechanisms by which chemicals cause cancer and the molecular changes that lead to tumor progression (Loeb and Harris, 2008). Thus, the next step, after the identification of chemical carcinogens in the environment and occupational settings related to cancer, is the generation of biological markers to assess altered metabolic pathways and the implementation of new targets for therapy (Loeb and Harris, 2008). For this, experimental carcinogenesis studies *in vivo* are essential. Fishes have being playing a role in this step, since they have received considerable attention as animal models for the study of chemical carcinogenesis among aquatic toxicologists and cancer biology groups. In addition to generating concern for the health of specific water populations, the epizootics of cancers in free-living fishes have presented themselves as useful "laboratories" for exploring chemically mediated carcinogenesis, in real-world complex scenarios that are relevant to human exposures (Rotchell et al., 2008).

Several small fish species have been excellent models for the screening of carcinogens that are very cost and time effective relative to standard *in vivo* rodent exposures. Biochemical and molecular mechanisms studies about the metabolism of carcinogens, DNA damage and repair, and oncogene activation in small and larger fish species, have revealed that many features of these processes are qualitatively shared among fish and mammalian models, though important, oftentimes species-specific differences have been observed as well (Rotchell et al., 2008). The rapid advancement of molecular biology is accelerating the elucidation of these similarities and differences, and their underlying basis. Relatedly, fish comprise definitely the most diverse class of vertebrates and thus provide a vast resource for studies in comparative biology (Rotchell et al., 2008).

While fishes have been proposed as valid models in cancer research, one remaining question is how similar are fish and human tumors at the molecular level. Fortunately, in a very recent study, Lam et al. (2013) performed a comparative analysis of microarray data from zebrafish liver tumors with those from four human tumor types, and the results were really interesting, revealing molecular conservation at various levels between the fish and human tumors. Therefore, based on gene ontology annotation of the putative human homologs mapped to Zebrafish Liver Tumor Differentially Expressed Gene (ZLTDEGS), a large number of genes coding for proteins involved in cell cycle/proliferation, apoptosis, DNA replication and repair, metastasis and cytoskeletal organization, protein synthesis and liver-specific functions were deregulated, indicating that the zebrafish liver tumors possess the general molecular hallmarks of human cancer. The results even suggest that there exist molecular similarities between zebrafish and human liver tumors that extends to tumor progression. In the end, this singular study provides strong molecular evidence highlighting the potential of zebrafish for modeling human liver cancer (Lam et al., 2013).

According to Rotchell et al. (2008) and the New Jersey Association for Biomedical Research (2012), the first event that triggered and supported the use of the trout as model was in early 1961, when an outbreak of liver cancer among middle-aged rainbow trout raised in fisheries that began to spread, bordering rapidly on epidemic proportions. The alarmed fish farm owners blamed everything from pesticides to water sources, and only later it was suspected about the food and realized that aflatoxins in pelleted hatchery foods were the cause of the liver tumors. Both humans and rainbow trout are susceptible to a byproduct of *Aspergillus* molds, the aflatoxin B1, a potent carcinogen, as previous cited. Only then scientists recognized the potential of this accidental discovery and these epizootics of liver neoplasms became the first instance in which the occurrence of fish tumors led to the recognition of a new family of chemical carcinogens. After this, world watched the beginning to a rapid succession of investigations of neoplasms in fish up to the present time.

Thus, if liver cancer could be consistently induced in rainbow trout after exposure to environmental carcinogens that also affect humans, like aflatoxin B1, it could effectively make liver cancer subjects more available for a wide a potentially wide range of studies. Therefore, the rainbow trout has been evaluated experimentally, being a highly sensitive animal for the detection and bioassay of the hepatocarcinogenic action of chemicals, all studies indicating that it is an ideal species for the analysis of the histo- and cytogenesis, additionally being perhaps, as recently investigated, a multi-organ carcinogenesis model (Rotchell et al., 2008; New Jersey Association for Biomedical Research, 2012).

Although being different from humans in many ways, rainbow trout, as other fishes, share key similarities in both patterns of gene expression and mutations that precede cancer and metabolic systems. There are several studies supporting this fact and that over the years have shown that many mechanisms important to carcinogenesis are similar in trout and mammals, e.g., pathways for metabolic activation, production of mutagenic DNA adducts, activation of oncogenes, etc. (Williams et al., 2003). Rainbow trout is actually viewed as an ideal model in liver disease because it presents advantages like: its relatively inexpensive character allows the significant increase of the study cases, since its purchase and per diem costs are a fraction of that spent with rodents; remarkable sensitivity to a number of human carcinogens; and, not less importantly, it has a low spontaneous tumor incidence (approximately 0.1%) (Williams et al., 2003; William et al., 2009). Besides AFB1, other environmental chemicals, like mycotoxins, polyhalogenated biphenyls (PCBs) and other aryl hydrocarbon receptor (AHR) ligands, promote liver cancer in trout, causing for instance, oxidative stress (Williams, 2012). Finally, natural estrogens, xenoestrogens ("xeno" means "foreign", so xenoestrogens refers to "foreign estrogens"), and phytoestrogens (plant-derived xenoestrogens) can be liver tumor promoters in this model as well (Williams, 2012).

In summary, the low husbandry costs, the quickly maturation, which facilitates the production of large numbers of fairly uniform animals, the high responsiveness and low background incidence of this trout tumor model makes it an excellent choice for conducting statistically challenging studies. Besides that, fish liver histopathology is a very useful biomarker of contaminant exposure and a notable tool in chemical toxicity and carcinogenicity testing, serving this animal model as environmental indicator as well as surrogate for human health problems (Dyk et al., 2012; Hobbie et al., 2012).

If the rainbow trout is a well-established model organism for experimental cancer research, the other common salmonids are not properly explored yet, such as the case of brown trout (*Salmo trutta*), originally an European species (Lerceteau-Köhler et al., 2013). Yet, this trout species is also readily available and is viewed as having a major economic and scientific relevance from a long time (Baglinière, 1999), besides it use as bioindicator

species or as experimental organism (e.g., Havelková et al., 2008; Valton et al., 2013). The first published data on liver carcinogenesis using brown trout appeared only recently (Santos et al., 2013), and so much remains to be explored with this promising “brown trout European cancer model” that could complement the “rainbow trout USA cancer model”.

## Influence of xenoestrogens in cancer and specifically in liver cancer

Over the past years, environmental health and oncology have demonstrated an increasing interest in estrogens as evolutionary conserved molecules, since, thanks to its endocrine, paracrine, and neurotransmitting activity, estrogens role is not limited to the regulation of the reproductive system. The distribution of estrogens receptors in mammalian tissues suggests that it could have a significant role in regulating a number of pathways during growth, differentiation and development (Fucic et al., 2012). This fact is a consequence of the estrogens capacity to regulate transcriptional activation of several molecules involved in key cellular processes such as generation of immune responses, cell proliferation and apoptosis through functional receptors localized in various subcellular organelles (Kalra et al., 2008). Additionally, these steroid hormones are critical regulators of various physiological processes in other tissues, so called nonclassical estrogen targets (bone, liver, kidney, cardiovascular system, spleen, lung, and brain). The majority of the biological estrogens action in humans and other vertebrates is mediated by two distinct, differentially expressed intracellular receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), which are considered uncommonly receptors, having affinity with environmental contaminants and pharmaceuticals (Benninghoff and Williams, 2008). The composition of estrogens includes a total of nine chemically different steroids, being the three major ones the 17β-estradiol (E2), estrone (E1), and estriol (E3) (Chang et al., 2011).

Every day, during their normal routine, population is exposed to a number of hormonally active compounds, introduced in the living environment in the last few decades, being the majority of them xenoestrogens. Polycyclic aromatic hydrocarbons (PAH), pesticides, polychlorinated biphenyl (PCB), dichlorodiphenyl-trichlorethane (DDT), some drugs, cotinine, phytoestrogens, mycotoxins, bisphenol A, phthalates, alkylphenols, and metalloestrogens are the examples of some chemicals that mimic oestrogen action, affect its levels, or bind to its receptors (Yardley, 2004). So, this leads to the possibility of these estrogen mimickers interfere with the natural and normal circulating level of estrogens; disrupt hormone balance and menstrual cycles; affect prostate health; contribute to problems with fibroids, endometriosis, uterine cysts, and polycystic ovary syndrome; and damage ova and sperm (Yardley, 2004).

Xenoestrogens are also present in a number of substrates such as cigarette smoke, automobile exhaust, chemical industry pollutants, grilled meat, volcano dust, forest fire smoke, milk, water, and cosmetic products (e.g., parabens, cyclosiloxanes) (Fucic et al., 2012). Some of these products are not biodegradable and remain in our environment for long periods of time, and the danger is its introduction into the food chain and eventually its consumption by larger animals and humans (Yardley, 2004). Several animal species have been suffering from reproductive complications over years, which clearly assume high proportions in polluted areas (Fucic et al., 2012; Ptak et al., 2013). As to humans, individual jobs can put them in risk because in certain occupations they are more likely to come into contact with and absorb specific toxicants: carpenters, auto mechanics, photographers, staff in the clothing and textile industry, workers involved in manufacturing synthetic hormones, laboratory technicians, commercial fisherman, painters, furniture workers, dentists, farmers administering hormones to livestock, electrical workers, potters and radiologists (Yardley et al., 2004).

Human and animal exposures to xenoestrogens have been associated to liver cancer, besides the reproductive system implications, with the development of abnormalities and risk increase of breast and ovary (Benninghoff and Williams, 2008). In fact, there are new evidences confirming xenoestrogens strong impact on carcinogenesis, by its involvement in key cellular processes such as apoptosis, cell cycle, proliferation, oxidative stress and inflammation (Kalra et al., 2008; Fucic et al., 2012). Therefore, xenoestrogens can play a critical role in the etiology since the binding to the ERα/β receptors, amplifies signals in either genomic, nongenomic, or mitochondrial ER-mediated signaling pathways that lead to increased cell proliferation and inhibition of apoptosis and consequently to uncontrolled cell division and tumor promotion (Chang et al., 2011). In other hand, the products of estrogen metabolism, mainly occurred in the liver, damage DNA by forming adducts and oxidized bases, provoking mutations in oncogenes and tumor suppressor genes that normally control cell growth and proliferation (Ptak and Gregoraszczuk, 2013).

As described by Ptak and Gregoraszczuk (2013), the classic estrogen receptors (ERs) are nuclear hormone receptors that act as transcription factors, regulating genes implicated several processes like homeostasis, development and metabolism. The classic mechanism of ER action implicates the binding of ER to its ligand, leading to the receptor dimerization, interaction with consensus estrogen-response elements (EREs), and recruitment of transcriptional co-regulators, which results in the formation of a complex that modulates the transcription of estrogen target genes. Microarray studies have been identified numerous genes with diverse functions in energy production, cell growth, cell cycle regulation, and cytoskeleton organization, whose expression is induced or repressed by estrogen (Ptak and Gregoraszczuk, 2013). Because of its ability to regulate gene expres-

sion, estrogens can function as a potent stimulus for proliferation and inhibition of apoptosis, which may lead to the development of cancer, including HCC, where assumes a relevant role in accelerating hepatocarcinogenesis and tumor progression. The mechanisms may vary, and may involve the Bcl-2 family of proteins, which regulates one of the key steps in the conserved apoptotic pathway; among the members of this family, Bcl-2 and Bcl-xL act as inhibitors of apoptosis. Ethinyl estradiol (EE2), a common component of OCs because it is a synthetic mimic of the major endogenous estrogen in humans, i.e., 17β-estradiol (E2), is known to increase the levels of Bcl-2 protein in cultured female rat hepatocytes (Kalra et al., 2008; Ptak and Gregoraszczuk, 2013).

Moreover, ER signaling can also result from a ligand-dependent, non-genomic (extra-nuclear) way, which involves the activation of other signal transduction pathways that leads to fast responses to estrogens exposure. This mechanism is not yet fully understood, but is potentially mediated by a membrane-associated receptor and can involve the activation of the mitogen-activated protein kinase (MAPK) or phosphoinositide-3-kinase (PI3) kinase signaling cascades, fluctuations in intracellular calcium, or stimulation of cAMP production, being MAPK and PI3 pathways responsible for cell proliferation and cell survival (Ptak et al., 2013). Deregulations of cell proliferation, differentiation, and apoptosis may allow the emergence of mutations in proto-oncogenes and tumor suppressor genes to survive and expand clonally (Ptak and Gregoraszczuk, 2013).

This possible convergence of genomic and non-genomic actions at multiple response elements provides an extremely fine degree of control for the regulation of transcription for ERs and the crosstalk between the genomic ER signaling cascades and kinase transduction pathways has a significant implication as a valuable therapeutic target to control ER-mediated cell proliferation and tumor growth (Chang et al., 2011).

In addition to genomic and non-genomic actions of estrogen mediated by nuclear and membrane ER, mitochondria has also recently been identified as important target of estrogen and ERs, since both ERα and ERβ have been reported to be present in the mitochondria of human HepG2 cells (Kalra et al., 2008). The mitochondrial genome has been shown to contain sequences that have partial homology to the estrogen responsive elements and results even suggest that estradiol is directly involved in the regulation of mitochondrial RNA transcription. The regulation of apoptosis and oxidative metabolism by estrogens in mitochondria also undertakes a role in the normal liver and in the development of HCC (Kalra et al., 2008).

Regarding the genotoxic effects via oxidative estrogen metabolism, Chang et al (2011) referred that the estrogen metabolites react with DNA leading to the mutations responsible for the initiation of cancer, being quinoids and hydroxyl radicals generated during the oxidative estrogen metabolism responsible for inducing oxidative DNA damage.

Thus, as cited earlier, there is a possibility for the involvement of OCs in liver neoplasia, since nuclear ERs are present in the hepatocytes and are increased in HCC, pointing for a hormonal responsiveness of hepatic neoplastic tissue (Maheshwari et al., 2007). In this context, estrogens may cause liver neoplasia by increasing proliferation and spontaneous mutations rates. Estrogenic direct genotoxicity is also possible because physiologically achievable concentrations of estrogen or estrogen metabolites have been shown to generate reactive oxygen species (ROS), which can increase genomic instability (Okoh et al, 2011). Its metabolites, quinones, cause the formation of DNA adducts, depurination, lipid-derived aldehyde-DNA adducts, and aneuploidy (Maheshwari et al., 2007; Fucic et al., 2012).

Additionally, recent *in vitro* study also suggests the ER involvement in many cellular events both connected as well as in interaction with viral proteins in hepatitis B virus (HBV) and hepatitis C virus (HCV)-induced HCC (Kalra et al., 2008).

***Estrogens: the other side of the coin***

As emphasized by Shimizu and Ito (2007), clinical observations and death statistics support that chronic hepatitis C and B seem to progress more rapidly in males than in females, and that cirrhosis is mostly a disease of men and postmenopausal women. Differences in the social environment and lifestyle of men and women may be involved in the basic mechanisms underlying the sex-associated differences of these chronic liver diseases, since, in general, it is more probably that men come into contact with a hepatitis virus and have an problematic addiction to drinking. Those authors also affirm that even environmental features of the male gender may lead to a greater preponderance towards nutritional and exercise problems for men. However, it cannot be forgotten that some mechanisms of the sex-associated differences may be based on biological factors, including estrogenic effects of female sex hormones, rather than on gender differences in the social environment and lifestyle.

In contrast with the data stated above, other studies (Mendelsohn and Karas, 1999; Omoya et al, 2001; Tian et al, 2012) highlighted the protective role of estrogens in several pathologies, including HCC. They suggested that estradiol treatment, for instance, can reduce hepatic steatosis and restore the impairment in mitochondrial and peroxisomal fatty acid β-oxidation in aromatase-deficient mice which lack intrinsic ability to produce estrogens. Furthermore, estradiol treatment was also shown to result in a dose dependent suppression of hepatic fibrosis in hepatic fibrosis models of male rats (Kalra et al., 2008).

It was noteworthy to Shimizu et al. (2007) that E2 and its derivatives (namely 2-hydroxyestradiol) are strong endogenous antioxidants that reduce lipid peroxide levels in the liver and serum. In the work, those authors suggest that E2 may protect hepatocytes from oxidative damage, inflammatory cell injury and cell death by induction of Bcl-2 expression, and by preventing macrophage accumulation and inhibiting proinflammatory cytokine production. In short, the study demonstrated that E2 production and ER status may play a role in hepatic defense and even have beneficial properties on the progression of chronic liver disease. However, it should be noted that, for further experiences, the administration of E2 *per se* in women poses some potential risks, including breast cancer and endometrial abnormalities, for the individual but also even for offspring generations initially noted more recently emphasized too (Shimizu et al., 2007; de Assis et al., 2012).

Form the above, it seems crucial to proceed with the liver studies in the context of sex hormones and their receptors and understand the true role of the estrogens in hepatic disease and what differentiates the carcinogenic effect from the protective roles.

## PRE-NEOPLASTIC LIVER LESIONS - FOCI OF CELLULAR ALTERATIONS (FCA)

In 1935, Sasaki and Yoshida were the first ones to verify that FCA precede the occurrence of chemically induced liver tumors and ever since that they have been centers of interest. FCA have been considered as the earliest emerging distinct phenotypic parenchymal changes indicating carcinogenic response in chemical liver carcinogenesis. Therefore, these preneoplastic lesions have no neoplastic nature but are capable to increased risk of developing into either benign or malignant tumors through an ordered sequence of phenotypically distinct lesions, and may be used to determine thresholds of effects of both genotoxic and non-genotoxic carcinogens (Enzmanna et al., 2013).

Banasch et al. (2003) sustained that there is not any hepatocarcinogenic agent which does not elicit FCA (in the original named foci of altered hepatocytes – FAH), nor is there any model of hepatocarcinogenesis without formation of these lesions prior to the manifestation of benign or malignant hepatocellular neoplasms. This presumption is based on the phenotypically similar preneoplastic lesions identified in human chronic liver diseases associated with, or predisposing to, HCC, irrespective of their etiology including chronic viral infections and a few defined chemicals. The striking similarities in the phenotype of FCA across all species investigated appears to be of relevant advantage for both mechanistic studies and the extrapolation of observations in animals to human beings besides the well-known obstacles in interspecies comparisons of toxicodynamics, toxicogenomics, and pathobiological processes. Lesions that resemble FCA have been observed fortuitously in the liver of women with long-term use of oral contraceptives and in

other pathologic conditions, particularly in the context of genetic hemochromatosis (Su et al., 1997; Banasch et al., 2003).

The detection of phenotypically similar FCA in several animal models and in the human liver does not only favors extrapolations from data across species, but, in our view, also opens new perspectives for secondary prevention of human HCC. Anyway, FCA as exactly seen in rats and other are now resonated as counterparts of human liver cell dysplasias, named as small *vs* large cell changes (Thoolen et al., 2012). Thus, besides the discovery of risk factors for neoplastic development, which is a prerequisite for primary prevention of cancer, the detection of preneoplastic alterations in the human liver is a premise for new approaches to secondary prevention of hepatic cancer (Banasch et al., 2003).

Hepatic FCA in fishes are included in a range of toxicopathic neoplastic, putative pre-neoplastic, and non-neoplastic lesions that have been studied in connection with the use of histological/histochemical biomarkers of toxic injury, dysfunction, and carcinogenesis (Feist et al., 2004). Considering that field and experimental studies have consistently demonstrated causal associations between exposure to xenobiotics and the development of FCA, along with other toxicopathic hepatic lesions, such lesion represent sensitive and reliable biomarkers not only of one individual condition but they more broadly often serve to assess the health of wild fish populations or even as proxies of the ecological status (Feist et al., 2004).

## FCA — Histological types

The continuous pathological process of hepatic neoplasia leads to the proliferation acceleration and formation of monoclonal populations: this is the hallmark of all preneoplastic lesions and takes place also in HCC. Thus, liver FCA represent the first discrete step, morphologically identifiable, in the possible evolution to carcinoma, since it is composed by differentiated hepatocytes that have acquired molecular aberrations and changed their metabolic properties, a kind of instability that may allow the progress to malignant, as mentioned before (Avellini et al., 2013).

FCA stand out as aggregates of hepatocytes with abnormal morphology and staining characteristics, which differentiate them from the surrounding parenchyma. In fish — and following the same general criteria as defined for rat and mouse — all categories can be (at least in theory) recognized using the following general morphological criteria (Feist et al.,2004; Koehler, 2004; Blazer et al., 2006; Thoolen et al., 2010; Laroche et al., 2013):

- Focal lesion of ten or more cells in diameter with no upper size limits;
- No evidence of compression of the surrounding tissue;

- Continuity of the tubular-like structure with the surrounding hepatic parenchyma and normal trabecular architecture;
- Trabecular aspect, due to the increasing of the vascularity and/or neoangiogenisis;
- Mitotic figures, macrophage aggregates, exocrine pancreatic tissue and bile ducts are were or absent;
- Features of cellular atypia (particularly increased nuclear to cytoplasmic ratio, nuclear pleomorphism, nucleolar enlargement, or even the presence of coarsely clumped chromatin) are not generally evident.

Lesions fulfilling the above morphological criteria can be further distinguished by variations in their cytological appearance and staining properties in hematoxylin and eosin stained (H&E) sections. Typically, authors divide the foci in clear, vacuolated, eosinophilic and basophilic, irrespective of nuances and other potential classifications. Authoritative descriptions of FCA can be found, e.g., in Boorman (1997), or Feist et al. (2004), or Koehler (2004), or Blazer et al. (2006). Based on these works, in the Sections below we summarize the relevant features.

***Clear cell foci***

Clear cell foci consist of hepatocytes with diffusely pale and finely vacuolated cytoplasm, which can be recognized by the “ground glass” appearance. This is indicative of increased glycogen storage within these cells. Hepatocyte nuclei in these foci are characteristically centrally placed. Some authors also reported prominent cell membranes, which can be confused with cytoplasmic content pressed to the periphery because of the vacuoles.

Occasionally, in these lesions the cytoplasm may be slightly eosinophilic, making it difficult to distinguish them from an eosinophilic focus. The Periodic acid-Schiff (PAS) technique, which demonstrates glycogen and other mucopolysaccharides, may be useful for the correct diagnosis of certain clear cell foci. However, it should be remembered that glycogen may be partially lost through the normal histological preparation process. Clear continuity of the foci cells with the surrounding parenchyma is often difficult to visualize in this lesion type, due to the constituent cells being frequently enlarged with cytoplasmic vacuoles.

Apropos, it is relevant to point that the role of clear cell foci in hepatocarcinogenesis is elusive and poorly described, although metabolic changes in carbohydrate metabolism have been associated with HCCs in both humans and rodents (Thoolen et al., 2012).

***Vacuolated foci***

Vacuolated cell foci are round to irregular, blend into the surrounding parenchyma without compression, and contain hepatocytes with clear lipid cytoplasmic vacuoles of varying sizes. As described above for glycogen, most lipids are lost during routine histological processing but can be demonstrated using frozen sections stained with (e.g.) Oil Red O. Because cytoplasmic lipids are often present in a microvesicular form, at low magnification the vacuolated foci may resemble the clear ones. As in these, the continuity of vacuolated foci with the surrounding liver parenchyma may be difficult to discern. Unlike hepatocytes in clear cell foci, the nuclei of hepatocytes in vacuolated foci tend to be displaced eccentrically to the cell margins.

***Eosinophilic foci***

As described by Feist (2004), the slightly enlarged — despite no quantitative study about this was made — and polygonal hepatocytes in this foci have affinity for eosin (acidophilic staining), with their constituent hepatocytes appearing pale to dark pink in H&E stained sections. This staining depends on the quality of the fixation of the tissue and the degree of differentiation used during the staining procedure. Although the degree of eosinophilia can vary, the foci should always appear relatively more eosinophilic than the surrounding tissue. The cytoplasmic eosinophilia is indicative of proliferation of the smooth endoplasmic reticulum within affected cells. There is little or no variation from normal cytomorphology and there is an absence of basophilic cytoplasmic fibrils. The works cited above for the other type of foci offer authoritative descriptions.

***Basophilic foci***

Basophilic foci form round to irregular clusters, and in some lesions constituent cells may be smaller than adjacent ones. The basophilia of the altered cells is due to the proliferation of rough endoplasmic reticulum and the cytoplasm is densely crowded by free ribosomes. Although the degree of basophilia can vary, as for the eosinophilic foci, the hepatocytes of the basophilic foci should always appear relatively more basophilic than the surrounding tissue. Regarding the adjacent unaffected hepatic tissues, there is no evidence of compression. Moreover, the observed of hepatocytes atypia is slight or inexistent, and mitotic figures are generally absent.

***Amphophilic foci***

These foci contain hepatocytes with both eosinophilic and basophilic tinges in the cytoplasm.

## The progression and fate of FCA

After the emergence of FCA, the subsequent step (morphologically defined) is represented by the formation of dysplastic nodules. In these lesions, it is probable that genetic and epigenetic mutations lead to a proliferative advantage for altered hepatocytes and to the involvement of several regulatory pathways in simultaneous disorders favoring the passage from dysplastic to neoplastic lesion. This way, the subsequent step is the development of hepatocellular carcinoma, which can be further classified into well differentiated, moderately differentiated and poorly differentiated tumors (Avellini et al., 2013; Youness et al., 2013).

Regarding the evolution of FCA themselves, Banasch et al (2003), Koehler (2004) and Enzmanna et al. (2014) defended that the process implies a sequential morphological changes of hepatocytes during the early phase of carcinogenesis. The earliest stage was proposed to be the vacuolated/eosinophilic foci (glycogenotic clear and acidophilic hepatocytes, showing a rich smooth endoplasmic reticulum). Consequentiality, supposedly occur a transition to, or new formation of basophilic foci (glycogen-poor and homogeneously hepatocytes, rich in ribosomes). In these proposed sequence of events, the vacuolated and eosinophilic foci are precursor lesions of basophilic foci. Furthermore, the appearance of a basophilic cell type is a preneoplastic stage, because this cell type persists during cancer progression and is the main cell type in hepatocellular carcinomas. Summarizing, there is evidence that an ordered sequence of phenotypically distinct lesions leads from clear to basophilic cell foci and finally to tumors. However, from fish to humans there is still controversy about the correct kinetics and fate of the pre-neoplastic hepatic changes (Thoolen et al., 2012; Santos et al., 2013); thus calling for more studies.

## OBJECTIVES

1. To confirm the triggering of liver carcinogenesis in brown trout by the use of a simple assay, based on bathing eye-egged embryos for 1 hour in solution of a reference genotoxicant (MNNG), either or not followed by exposure to presumptive promoters;

2. To confirm the hypothesis that estrogenic stimuli can act as promoters of (at least) liver pre-neoplastic lesions in brown trout, by chronically exposing initiated fish either to 17α-estradiol or to a mixture of two industrial xenoestrogens of the alkylphenols family;

3. To diagnose the lesions that appeared after the experiment of initiation followed by exposure to estrogens, and to estimate the sample prevalence of the lesions in each experimental condition, for concluding about any sort of differential promotion effect;

4. To implement a stereological study targeting selected parameters that could clarify the hypothesis that the different experimental conditions tested would induce various outcomes, both as to the relative lesions extension in the liver and the structure of the hepatocytes and capillary network integrating the anticipated various types of lesions;

5. To start investigating the hypothesis that the onset of a liver neoplastic process, and presence of pre-neoplastic and/or neoplastic lesions, alter the parenchyma that seem otherwise normal, to the point of impacting on size-related parameters of hepatocytes;

6. To contribute to the continuous effort of generating data that may be useful for the differential diagnosis of the early lesions appearing during liver carcinogenesis in fish, namely by combining standard staining with histochemistry and immunohistochemistry;

7. Finally, as an additional aim, we take the opportunity of this study to implement a parallel diagnosis approach using conventional microscopy and whole digital slides, to see whether or not the digital histology could match or surpass the standard approach.

# MATERIALS AND METHODS

# MATERIALS AND METHODS

## EXPERIMENTAL SET UP AND ASSAY

Hepatocarcinogenesis induction was made in brown trout (*Salmo trutta* f. *fario*) embryos, and used *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) as the initiator agent. In summary, eyed-eggs were waterborne exposed to 50 ppm of MNNG (1 hour). Fish embryos were then kept in standard hatching-incubator troughs for salmonids, under routine husbandry conditions for trout, implying manual removal of any dead embryo, tank cleaning and continuous pristine freshwater supply. Eight-weeks later, 4 fish groups were exposed (for 8 months) to the next nominal conditions: 1) 0.001% of ethanol saline (used as dissolving vehicle for the other tested compounds) 2) 5 µg/L of 17β-estradiol (E2); 3) 50 µg/L of E2; 4) 500 µg/L of alkylphenol mixture (250 µg/L of nonylphenol + 250 µg/L of butylphenol). One additional group was formed, not exposed neither to MNNG (despite being subjected to the exact same manipulation, but only being exposed to water) initially nor to any other compound; this made a complete blank control. All reagents used were from Sigma-Aldrich.

Each condition was run in duplicate, in well-aerated tanks with dechlorinated high quality filtered groundwater, closing the water-circuit, but keeping water circulation with a pump and with the water continuously passing by sponge filter to remove particulate matter. Water parameters were monitored every other day to confirm they were under good conditions for salmonids (Salmonid Water Quality Standards, 1988; Klontz, 1991). The water was fully renewed every 48 hours, taking the opportunity to reconstitute the nominal concentrations of each tested compound (inc. in the vehicle only group). In the end, and after being captured with a fishnet, every fish was painless euthanatized with an overdose of ethylene glycol monophenyl ether, within a well aerated 5 L plastic bucket. The fish from the different groups did not differ in mass, weighing about 19 g (CV = 0.60).

The assay was made according to the European legislation, following guidelines of the European Union Council and the national law.

## HISTOLOGY

After death, each animal was immediately dissected to extract the liver. After excision, the organ was sliced into 2-4 mm thick slabs that were fixed for 24 h in 4% buffered formaldehyde. Then, the slabs were routinely processed for paraffin embedding, sec-

tioned (≈ 4 µm in thickness) in a rotary microtome, stained with hematoxylin–eosin and mounted with DPX (Sigma-Aldrich) for histopathological classification of the FCA, estimation of their prevalence, and register of eventual other abnormal features. Slides were studied at light microscopy (without the observer knowing the group) and, for archiving selected representative lesions, images were taken with a color cooled digital microscope camera.

## QUALITATIVE ANALYSIS AND STEREOLOGY

For qualitative assessment, a blind assay was implemented, where the observer (Sara Pereira) did not know neither the slides identification nor to which group they belonged. The analysis was in two phases, being the first essential to pursue the second one:

(1) In the first one, using the optical microscope, the livers were classified regarding the (1.1) seemingly normal parenchyma (basophilic, intermediate or vacuolated), (1.2) number and phenotype of FCA (basophilic, amphophilic, eosinophilic, clear and vacuolated cell), which were judged as FCA only when having the characteristics described in the Introduction of this Dissertation, including having at least 12 cells observable, and (1.3) preferential localization (none, perivascular or peripheral);

(2) In a second stage, the FCA were reanalyzed, concerning their type, targeting also pre-lesions that, according to our criteria, could not be properly classified as FCA (see Results), measuring the area of each and every lesion against the total area of all the sections used per animal; by the Delesse principle, the relative areas are typically unbiased estimates of the relative volumes ($V_V$) (Howard and Reed, 2005).

The measurement of the areas was executed through scanned slides, which resulted from the utilization of the Olympus VS110 virtual slide scanning system. Before loading the slides into the device, they were carefully clean (because any residue could interfere with the focus plan) and, consequently, with the final resolution of the digital image. (One of the greater advantages of the used model is the 100-slide robotic loader for automated scanning, so the slides can be selected sequentially and an integrated barcode scanner ensures that metadata is automatically loaded and linked with each slide's virtual image. This particularity allowed us to program the machine for scanning overnight.) All the slides previously diagnosed were scanned and all the sections of vehicle control, alkylphenols and E2 5 µg/L groups were analyzed, measuring both the total area and FCA areas. Regarding the E2 50 µg/L group, and facing the higher frequency of lesions, despite all slides were scanned, the sections to be measured were systematically selected (with a random number for picking the first section); typically, half of the sections were sampled for measurements of areas. These were made with two tools: a) free hand polygon (the operator circumvents freely the area, used for the FCA); and b) magic hand (the

operator clicks in the section and the program automatically circumvents it, obtaining easily the total area). The values were exported to Microsoft Excel for required computations.

## NORMAL PARENCHYMA: WITHOUT LESIONS *VS* WITH LESIONS

Another fundamentally interesting studied aspect herein was the search for eventual differences between the hepatocytes making the normal parenchyma of animals without lesions (control group that was not exposed to MNNG) and those making the seemingly normal hepatocellular parenchyma areas of fish with lesions (belonging to the remaining four groups). Three stereological parameters for sizing were estimated: (1) number-weighted volume of the hepatocyte - $\bar{v}_n$ (hepatocyte), (2) number-weighted volume of the hepatocyte nucleus $\bar{v}_n$ (nucleus), and (3) volume-weighted volume of hepatocyte nucleus – $\bar{v}_v$ (nucleus). In all cases, we used a systematic sampling approach in the selection of the fields for the stereological analysis, which was performed with a workstation that comprised a microscope (BX-50, Olympus equipped with a 100× oil immersion objective (NA 1.30, UPlan, Olympus), a CCD video camera (Sony) connected to a PC monitor and a motorized stage (Prior) for stepwise displacements in x–y directions; the workstation was controlled by the software CAST-Grid (Version 1.5, Olympus, Denmark).

The $\bar{v}_n$ (hepatocyte) and $\bar{v}_n$ (nucleus) were estimated using the nucleator in one thin section method (Moller et al., 1990), previously validated for our material under study (unpublished data). The nucleator method uses a fixed point which is sampled using the disector principle (Sterio, 1984), which gives every particle no matter the size or shape an equal probability of being sampled. It is an efficient way to estimate the number-weighted mean volume, possessing the particles a single identifiable point-like inclusion — at the cellular level, an ideal inclusion is the nucleolus. In this technique, an unbiased estimate of the number-weighted mean volume is derived, based on one or two isotropic lines that are drawn from the nucleolus to the nuclear or cellular border (central point to the particle boundary) (Mayhew, 1992; Mayhew and Gundersen, 1996; Marcos et al, 2012). We used herein a simplified procedure ("single-section nucleator"), based on the nucleator (with two lines), in all liver cell for which the nucleolus (typically one) was evident in the thin section. The adopted sampling step for the inherent meander sampling was X,Y = 900 µm, in areas of normal parenchyma, it was X = 60 µm and Y = 40 µm in smaller FCA (up to a sectioned area of ≈ 80 000 µm$^2$), and, at last, it was X = 120 µm and Y = 80 µm in other FCA. From measurements of the isotropic lines, volumes were estimated by the next formula:

$$\bar{v}_n = \left(\frac{4\pi}{3}\right).\bar{l}_0^3$$

The $\bar{v}_v$ (nucleus) was estimated by the point sampled intercept method (Gundersen and Jensen, 1985). This parameter is useful for analyzing certain structural changes of pathological processes, as alterations in the size and/or size distributions of cells and their nuclei (Mayhew, 1992). Thereby, the method simultaneously quantifies the nuclear size and pleomorphism, technically involving: sampling the particles (cells or nuclei) according to their volume, by overlaying a grid of points at random; drawing of a line from the point to the particle border, in an isotropic direction, for each particle hit by a point; drawing of a second line in the opposite direction and measuring of both distances using a rule, in a linear or a non-linear scale.) For the areas of normal parenchyma the sampling step was set to X,Y = 1000 μm, for the small FCA (up to a sectioned area of ≈ 80 000 $\mu m^2$) the setp was X = 60 μm and Y = 40 μm, and, finally, for bigger FCA the step moment was X = 120 μm and Y=80 μm. Herein the all procedure was semi-automatically implemented via the cited CAST-Grid system, being the volumes derived by the next formula:

$$\bar{v}_v = \left(\frac{\pi}{3}\right).\bar{l}_0^3$$

In the end, and in order to obtain information about the nuclear size variation, the coefficient of variation (CV) of the nucleus in the number-weighted distribution was given according with the following formula (Gundersen and Jensen, 1985):

$$\mathrm{CV}(v) = \sqrt{\frac{\bar{v}_v\,(\mathrm{nucleus})}{\bar{v}_n\,(\mathrm{nucleus})} - 1}$$

In addition to the above volumes and related parameters, we also estimated herein the relative volumes ($V_V$) — also named volume densities — of the nuclei of the hepatocytes and of the sinusoidal lumina in relation to the FCA. This estimates we made using a classical manual technique based on point counting (Thompson, 1930; in: Howard and Reed, 2005). For this we also used the mentioned CAST-Grid system, were a virtual grid with a total of 81 points was laid down over fields within the FCA (the reference space of the estimates). From all the grid point only 9 were used just for the reference space. The final estimates were computed using the following formula:

$$V_V\,(structure\ of\ interest, reference\ space) = \frac{\sum \mathrm{P(structure\ of\ interest)}}{\mathrm{k}.\sum \mathrm{P\ (reference\ space)}}$$

In the above formula, ∑P(structure of interest) corresponds to the point fallen over either the nuclei of the sinusoidal lumina, summed across all fields of view, ∑P(reference space) correspond to the points falling within the FCA, summed across all sampled fields, and k is the ratio of the two-lattice gird point (ie, 9:81 points, providing a k of 9). The meander sampling for the $V_V$ estimates followed a step of X = 60 μm and Y = 40 μm, for FCA up to a sectioned area of ≈ 80 000 $\mu m^2$, and X = 120 μm and Y= 80 μm, for larger FCA.

## STATISTICAL ANALYSIS

The descriptive statistics was made using the Microsoft Excel. For the inferential statistical analysis as to the prevalence of each type of FCA and FCA combinations per group we used the VassarStats: Website for Statistical Computation (http://vassarstats.net/); viz. the "Significance of the Difference Between Two Independent Proportions" test. All the subsequent analyses were made with the software STATISTICA 12. As to the analyses of the relative volumes, and because the data failed to show homogeneity of variances using the Levene test, we executed the non-parametric Kruskal–Wallis test to verify whether or not groups significantly differed. Planned comparisons of pairs of means were made with the Mann–Whitney U-test. Concerning the study of the parenchyma parameters, we perform a parametric analysis, resourcing to Newman-Keuls test, after every significant one-way ANOVA, to compare and identify means significantly different. In all tests we adopted the typically standard significance level ($\alpha = 0.05$).

## HISTOCHEMISTRY

The procedures were applied to cases selected for having diverse types of lesions. The fragments, routinely processed for paraffin embedding as described, were sectioned at 4 μm of thickness, the resulting sections being deparaffinized and hydrated. Then, the following histochemical stains were performed, as detailed below:

(1) Periodic acid-Schiff (PAS) staining: sections were oxidized with 0.5% periodic acid solution (10 minutes) and placed in Schiff reagent (15 minutes). PAS diastase (PAS/D) controls were implemented to confirm the presence of glycogen, treating the sections initially with amylase solution for 30 minutes at 37ºC. This latter technique in based on the following principle: alpha-amylase, also known as diastase, is an enzyme that cleaves the a-glucosidic 1-4 linkages of glycogen leading to the formation of maltose and dextrose (water-soluble sugars). So, when sections are pre-exposed to diastase before the PAS technique the glycogen within the tissue is broken down into maltose and dextrose, which are dissolved and washed away when the section is rinsed sufficiently in tap water. Subsequently, glycogen will be stained magenta on the PAS stained slide and will be absent on the PAS/D stained slide;

(2) Perls' Prussian blue staining: we placed the slides in a mixture of equal parts of 2% potassium ferrocyanide and 2% chloric acid, for 30 minutes at 60ºC, and then counterstained with eosin, with a rapid emerge in the dye;

(3) Sirius red staining: we stained first with azure and Mayer's haematoxylin and then with picrosirius red, for one hour.

Irrespective of the technique, in the end all the slides were dehydrated in absolute alcohol, cleared in xylene and mounted with DPX.

## IMMUNOHISTOCHEMISTRY

The procedures were applied to cases selected for having diverse types of lesions. Prior to immunohistochemistry, the sections with 4 µm of thickness were deparaffinized and hydrated. An antigen retrieval-microwave method was performed, using: (1) citrate buffer (pH 6), with a microwave at 600 W, during 2 min plus 2 x 4 min, for CYP1A immunochemistry; (2) Tris-EDTA buffer (pH 9.0), with a microwave at 600 W, during 10 min after boiling, for E-cadherin immunochemistry. Endogenous peroxidase was quenched by treatment with a 3% $H_2O_2$ solution in methanol, for 10 min. After rising in PBS with Tween 0.05%, it was performed an avidin and biotin blocking step (Vector Labs kit), followed by another rising with PBS and with BSA and then, to reduce non-specific binding, sections were incubated in a blocking solution (Histostain-Plus kit, Invitrogen), for 1 hour. As to the antibodies, sections were incubated overnight, at 4ºC, in a moist chamber, with the rabbit anti-fish CYP1A peptide polyclonal antibody, CP-226 (Biosense Laboratories) and monoclonal mouse anti-human E-cadherin, clone NCH-38 (Dako).The CYP1A and E-cadherin antibodies were used at a dilution of 1:200 and 1:100 and 1:50 respectively, in 0.01% phosphate buffered saline (PBS, pH 7.5). After rising in PBS and incubation with a broad spectrum secondary antibody (Histostain-Plus kit, Invitrogen) for 20 min, the sections were rinsed and incubated with Streptavidin-HRP conjugate (Histostain-Plus kit, Invitrogen), for 20 min. After subsequent rinsing in PBS, the sections were incubated with 3,3´diaminobenzidine (DAB) (Zytomed Systems) for 2 min, and counterstained with hematoxylin. In the end, all the slides were dehydrated in absolute alcohol, cleared in xylene and mounted with DPX.

# RESULTS

# RESULTS

## GENERAL QUALITATIVE FINDINGS

Fish from the blank (non-MNNG initiated) control did not have any sort of liver lesions. By the contrary, basophilic (Figure 1, A and B), amphophilic (Figure 1, C and D) and eosinophilic FCA (Figure 1, E and F) were found in all the MNNG initiated groups. Vacuolated (Figure 2, C and D) and clear FCA (Figure 2, A and B) were also found, however, as 21% of the animals presented a hepatocellular parenchyma quite broadly vacuolated, and 48% had an intermediate stage (i.e., cytoplasm between vacuolated and basophilic), the distinction between the foci and the normal tissue was very difficult and uncertain; so we decided to focus our attention in the remaining types and do not advance with further qualitative and quantitative analysis about vacuolated and clear FCA. These foci were not accounted in the statistical analysis and will be analyzed in a next phase of the all project. In addition to those well-defined FCA, irregular zones of identifiable cellular degeneration also existed, resembling FCA but being distinctly undefined as to the tinge and focal nature; thus being characterized by liver cells that had a different staining, but in which more than 50% of the margins of the "cell group" were poorly defined, having a very reduced or absent trabecular aspect, and, finally (contrary to is observed in well-defined FCA) without being associated with perceivable sinusoidal enlargement. We did not find mixed FCA (i.e., focal lesions with a region that could be seen as basophilic apart from another with eosinophilic aspect). During analysis we discriminate an early stage of FCA, a lesion that does not present yet all the features to be identified like FCA — such cases were called Pre-FCA (Figure 2, E and F); and its differential diagnosis will be discussed ahead. Besides FCA, we also diagnosed two cases of adenoma (Figure 3, A and B) in E2 50 µg/L group. We faced some difficulties in a few cases, derived from a generalized alteration, where either all the section or most of it seemed a huge FCA (Figure 3, C and D).

## PREVALENCE

All the raw data and the derived prevalences for the studied lesions are given in Table 1. Additionally, the significance of the differences between the groups is exposed in Table 2. Regarding the prevalence, the E2 50 µg/L group reaches a remarkable result, with 100% of fish having FCA, followed by the Alkylphenols group, then the Vehicle, and finally, the E2 5 µg/L group; these three with similar percentages. The group with the lowest % value is the E2 5 µg/L, with less than 50% of the animals detected with FCA.

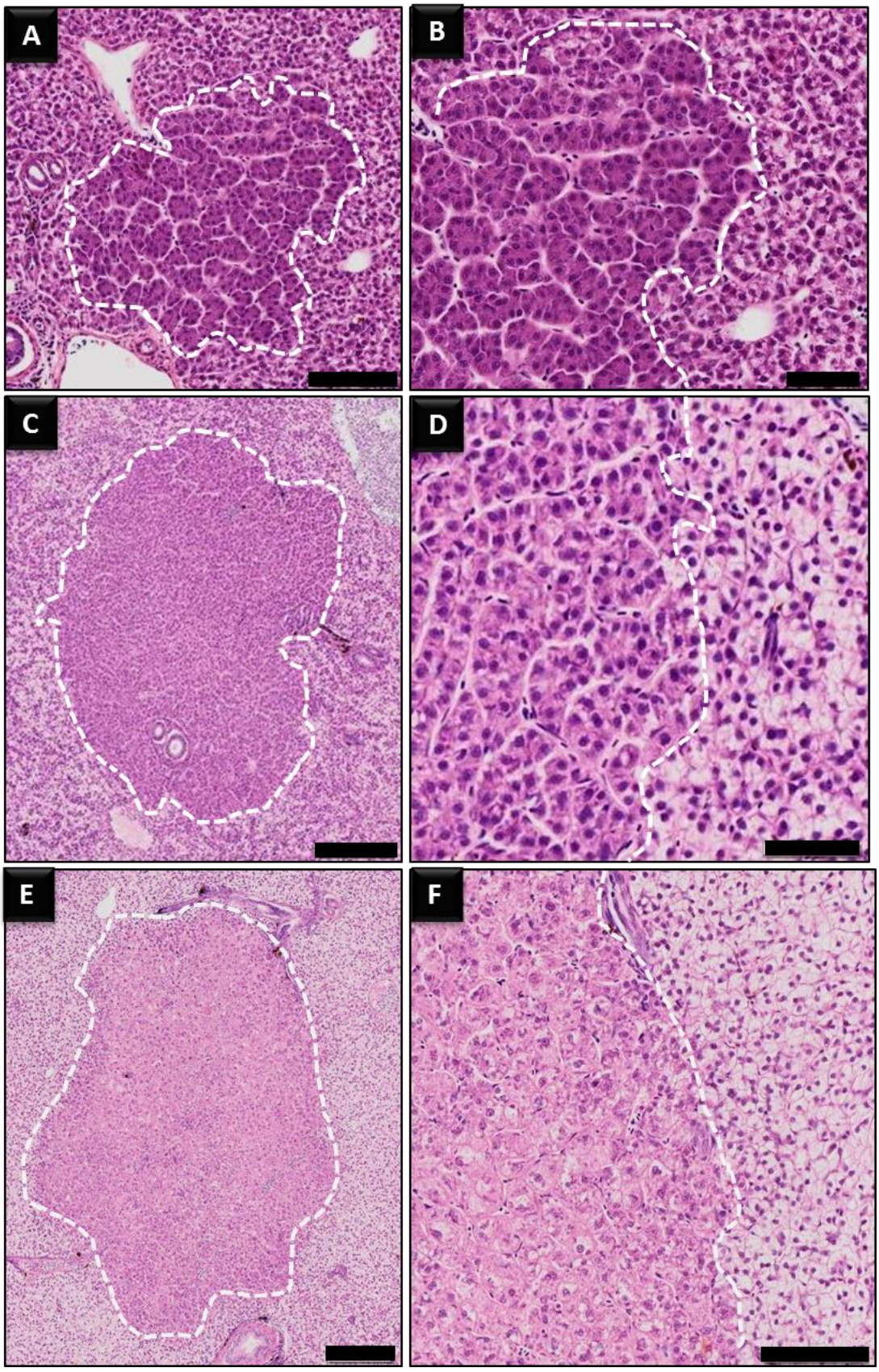


Figure 1 Hematoxylin and Eosin staining. Basophilic FCA (delimited in A and detailed in B); amphophilic FCA (delimited in C and detailed in D) composed of hepatocytes with cytoplasmic basophilia and eosinophilia; eosinophilic FCA (delimited in A and detailed in B), presenting an acidophilic staining. Bars: A- 100 µm; B- 50 µm; C- 200 µm; D- 50 µm; E- 200 µm; F- 100 µm.

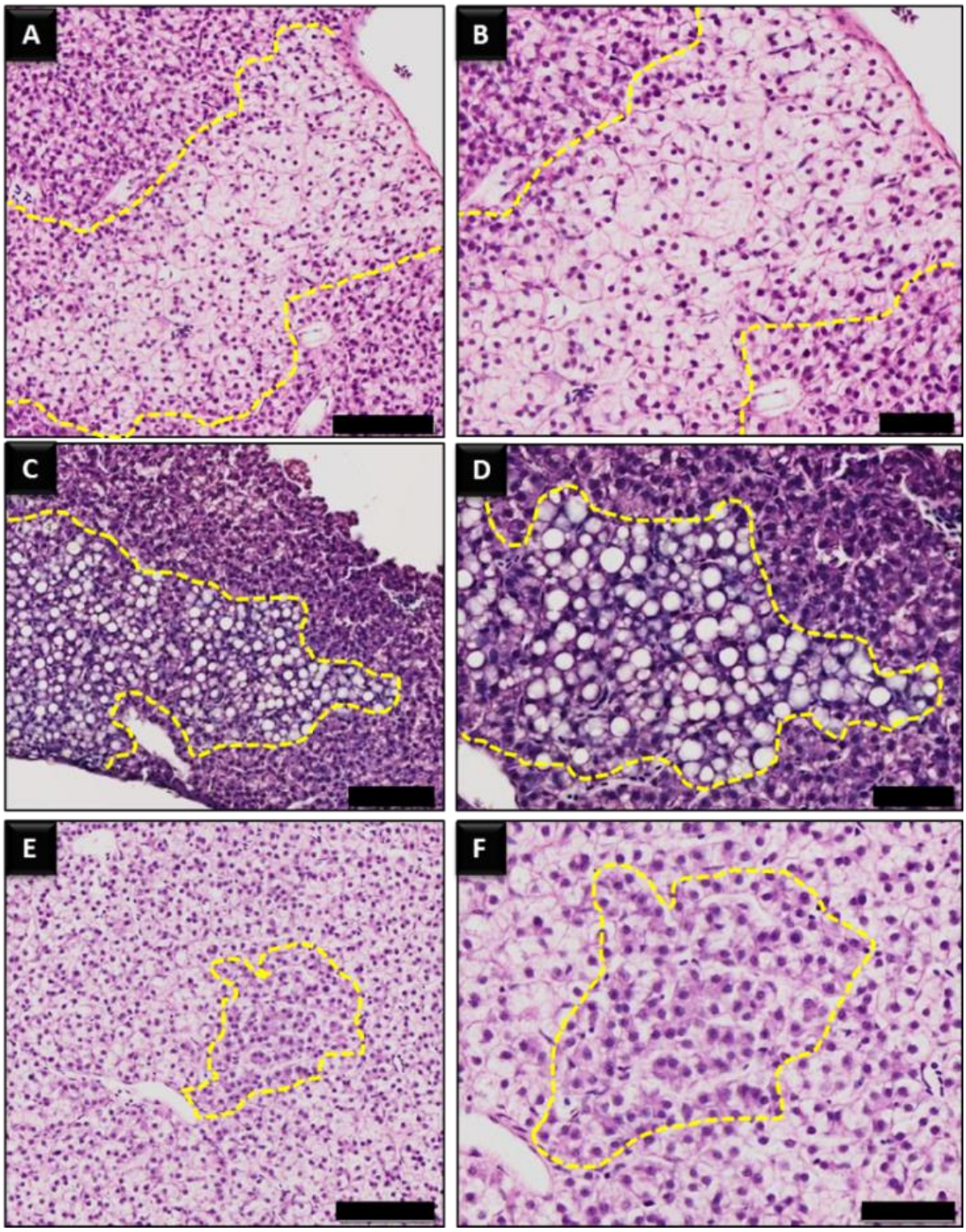


Figure 2 Hematoxylin and Eosin staining. Clear cell foci (delimited in A and in detailed in B) with hepatocytes with a faint pink colour and central nucleus; vacuolated FCA (delimited in C and in detailed in D) an evidently vacuolated cytoplasm and an eccentrically displaced nucleus; Pre-FCA (delimited in E and in detailed in F) with a mild tinctorial change regarding the hepatocytes but with neither a trabecular aspect nor increased vascularization. Bars: A- 100 µm; B- 50 µm; C- 100 µm; D- 50 µm; E- 100 µm; F- 50 µm.

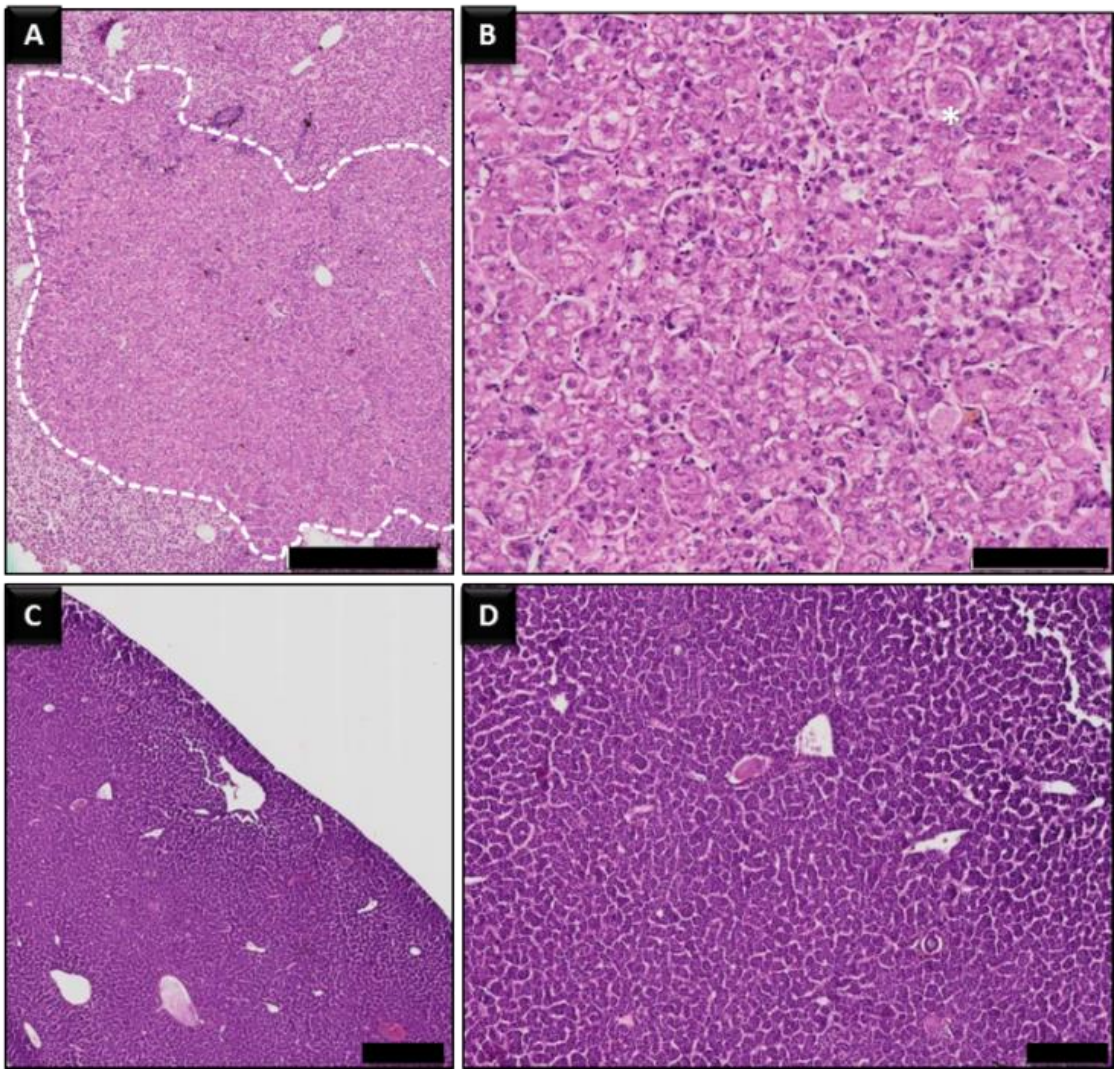


Figure 3 Hematoxylin and Eosin staining. Hepatocellular adenoma (delimited in A and in detailed in B), well delimited from the parenchyma, depicting evident cellular and nuclear atypia and pleomorphism (*binuclear cell). Liver displaying a widespread abnormal pattern with heterogeneity (C and in detailed in D), where all the parenchyma seems to have turned into an enormous FCA. Bars: A- 500 µm; B- 100 µm; C- 500 µm; D- 200 µm.

**Table 1- Prevalence of each type of foci of cellular alteration (FCA) and Pre-FCA in the different groups (fish, n=426).**

| Group | Fish (n) | Fish with FCA (n) | FCA (%) | bFCA (n) | bFCA (%) | aFCA (n) | aFCA (%) | eFCA (n) | eFCA (%) | Pre-FCA (n) | Pre-FCA (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **VEHICLE CONTROL** | 112 | 73 | 65% | 70 | 63% | 26 | 23% | 2 | 2% | 36 | 32% |
| **ALKYLPHENOLS** | 111 | 76 | 68% | 64 | 58% | 42 | 38% | 2 | 2% | 63 | 57% |
| **E2 5 µg/L** | 110 | 54 | 49% | 49 | 45% | 12 | 11% | - | - | 36 | 33% |
| **E2 50 µg/L** | 93 | 93 | 100% | 81 | 87% | 88 | 95% | 40 | 43% | 79 | 85% |

bFCA - Basophilic FCA. aFCA - Amphophilic FCA. eFCA - Eosinophilic FCA.

**Table 2- Significance of the difference (p-values) of the prevalences of foci of cellular alteration (FCA) and Pre-FCA between groups, under the VassarStats test.**

| | VEHICLE CONTROL | | | | | |
|---|---|---|---|---|---|---|
| **ALKYLPHENOLS** | FCA | 0.6017 | | | | |
| | bFCA | 0.4605 | | | | |
| | aFCA | **0.0177** | | | | |
| | eFCA | 0.9928 | | | | |
| | Pre-FCA | **0.0002** | **ALKYLPHENOLS** | | | |
| **E2 5 µg/L** | FCA | **0.0154** | FCA | **0.0034** | | |
| | bFCA | **0.0073** | bFCA | 0.0512 | | |
| | aFCA | **0.0149** | aFCA | **0.0017** | | |
| | eFCA | - | eFCA | - | | |
| | Pre-FCA | 0.9259 | Pre-FCA | **0.0003** | **E2 5 µg/L** | |
| **E2 50 µg/L** | FCA | **< 0.0002** | FCA | **< 0.0002** | FCA | **< 0.0002** |
| | bFCA | **< 0.0002** | bFCA | **< 0.0002** | bFCA | **< 0.0002** |
| | aFCA | **< 0.0002** | aFCA | **< 0.0002** | aFCA | **< 0.0002** |
| | eFCA | **< 0.0002** | eFCA | **< 0.0002** | eFCA | - |
| | Pre-FCA | **< 0.0002** | Pre-FCA | **< 0.0002** | Pre-FCA | **< 0.0002** |

Statistically significant differences ($p \leq 0.05$) in bold. bFCA - Basophilic FCA. aFCA - Amphophilic FCA. eFCA - Eosinophilic FCA.

In all groups, except the E2 50 µg/L, the most frequent FCA type is the basophilic, and the less common is the eosinophilic, being the E2 5 µg/L the only group without a register of this type of FCA. In the E2 50 µg/L group, eosinophilic is also the less frequent type, and it is the amphophilic that reaches the highest percentage. The E2 50 µg/L is also the group with a higher rate of Pre-FCA, followed by the Alkylphenols group, and then E2 5 µg/L and Vehicle Control groups; the latter three having very approximated frequencies.

Concerning the significance of the difference between two groups (Table 2), taking into account the prevalence of each type of FCA, in general we observed that every group differs from the E2 50 µg/L group ($p < 0.0002$). The Vehicle Control and the Alkylphenols groups did not differ from each other, except in the prevalence of aFCA and Pre-FCA, with the Alkylphenols group achieving higher (significant) numbers. The E2 5 µg/L differs from Vehicle Control and the Alkylphenols groups in every proportions of FCA, except in Pre-FCA, where the result points to a similarity with Vehicle Control group; it was not possible to compare the number of eFCA, since the E2 5 µg/L group did not present this type.

Another way to look at data is to organize the information as combined lesions (Graphs 1-4). So, about the prevalence of different FCA combinations, we can state that the percentage of animals with only bFCA reached the highest values in all groups, except in the E2 50 µg/L group, where bFCA + aFCA is the most frequent combination. Since the E2 50 µg/L group achieved 100% of fish with FCA, there were no fish with Pre-FCA only.

The Vehicle Control and E2 50 µg/L groups were the only ones that presented the aFCA + eFCA combination. The presence of all types of FCA — bFCA + aFCA + eFCA — in the same slide was observed in Alkylphenols and E2 50 µg/L groups. The E2 5 µg/L group did not register the bFCA + eFCA combination; however, all the remaining groups presented a single case of such a mixture. In all groups, the % of animals with detected aFCA only is 3 to 7%, except for the Alkylphenols group that had 15%.

Regarding the significance of the difference between two groups (Table 3), and considering the prevalence of FCA combinations in the four groups with lesions, the Vehicle Control and the Alkylphenols groups did not differ from each other, except in the number of fish with aFCA only. The E2 5 µg/L group did not differ from the Vehicle Control and from the Alkylphenols group, except in the number of fish with the combination bFCA + aFCA. The E2 50 µg/L group differed from all the other groups regarding the number of fish only with bFCA and with the combination bFCA + aFCA, but did not differ as to fish both with aFCA only and with the combination bFCA + eFCA. The Vehicle Control and E2 50 µg/L groups were the only ones that presented the combination aFCA + eFCA, and even so they differed from each other. The Alkylphenols and E2 50 µg/L groups were the only ones that had the combination bFCA + aFCA+ eFCA, and also differed from each other significantly.

**Graphs 1-4 - Prevalence of different foci of cellular alteration (FCA) combinations in the four groups.**

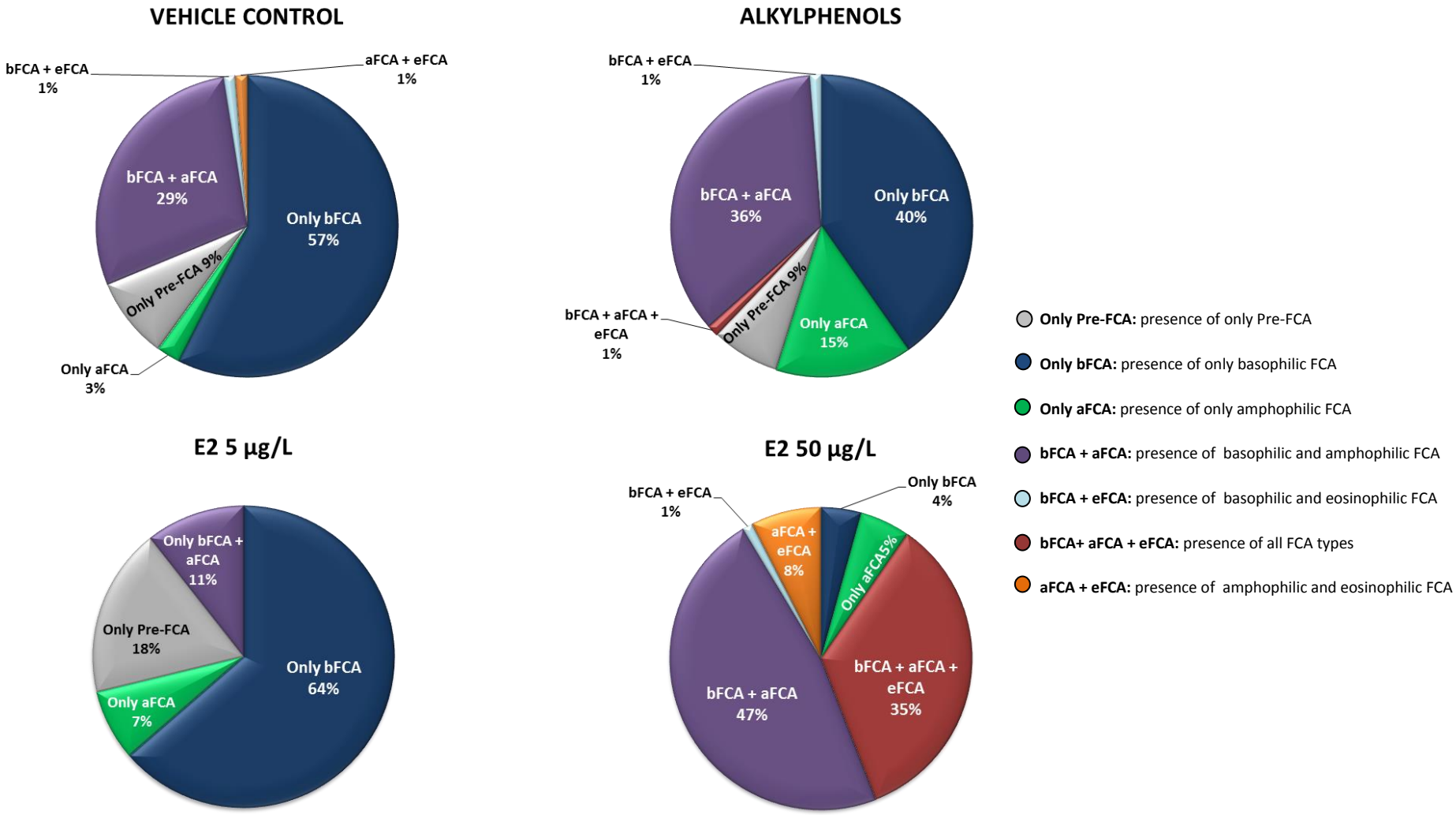

**Table 3- Significance of the difference (p-values) between groups, regarding the prevalence of FCA combinations, under the VassarStats test.**

| | VEHICLE CONTROL | | | | | |
|---|---|---|---|---|---|---|
| **ALKYLPHENOLS** | Only bFCA [a] | 0.0766 | | | | |
| | Only aFCA [b] | **0.0055** | | | | |
| | Only Pre-FCA [c] | 0.7897 | | | | |
| | bFCA + aFCA [e] | 0.3236 | | | | |
| | bFCA + eFCA [f] | 0.9952 | **ALKYLPHENOLS** | | | |
| **E2 5 µg/L** | Only bFCA [a] | 0.6599 | Only bFCA [a] | 0.1845 | | |
| | Only aFCA [b] | 0.2396 | Only aFCA [b] | 0.0805 | | |
| | Only Pre-FCA [c] | 0.2146 | Only Pre-FCA [c] | 0.1347 | | |
| | bFCA + aFCA [e] | **0.002** | bFCA + aFCA [e] | **< 0.0002** | **E2 5 µg/L** | |
| **E2 50 µg/L** | Only bFCA [a] | **< 0.0002** | Only bFCA [a] | **< 0.0002** | Only bFCA [a] | **< 0.0002** |
| | Only aFCA [b] | 0.1588 | Only aFCA [b] | 0.1618 | Only aFCA [b] | 0.7849 |
| | bFCA + aFCA + eFCA [d] | - | bFCA + aFCA + eFCA [d] | **< 0.0002** | bFCA + aFCA + eFCA [d] | - |
| | bFCA + aFCA [e] | **< 0.0002** | bFCA + aFCA [e] | **0.0017** | bFCA + aFCA [e] | **< 0.0002** |
| | bFCA + eFCA [f] | 0.8950 | bFCA + eFCA [f] | 0.8997 | bFCA + eFCA [f] | - |
| | aFCA + eFCA [g] | **0.0146** | aFCA + eFCA [g] | - | aFCA + eFCA [g] | - |

Statistically significant differences ($p \leq 0.05$) in bold. [a] Presence of basophilic FCA only; [b] Presence of amphophilic FCA only; ; [c] Presence of Pre-FCA only; [d] Presence of all FCA types; [e] Presence of basophilic and amphophilic FCA; [f] Presence of basophilic and eosinophilic FCA; [g] Presence of amphophilic and eosinophilic FCA.

## RELATIVE VOLUMES OF THE LESIONS

As we can see in Table 4, when comparing with the other lesions, the $V_V$ (bFCA) average reached the highest values in all groups, except in E2 50 µg/L group, where the $V_V$ (aFCA) was greater. Anyway, based solely on the maximum values, the aFCA were the foci that attained more extension in all groups. On the contrary, the eFCA were the foci less relatively voluminous, except, again, in the E2 50 µg/L group, where they even exceeded the bFCA as to the maximum value; but not on average. Concerning the $V_V$ both of all the FCA combined (Total FCA) and of the total altered parenchyma (TAP), i.e., considering both the Pre-FCA and FCA together, the E2 50 µg/L group reached more than the quadruple of the second highest group, in line with the previous results; that is, besides the prevalence of lesions, this group also excelled in extension.

**Table 4- Descriptive statistical data from each group, regarding the relative volumes (%) of the lesions, considering the liver as reference space (fish, n = 426).**

| | | VEHICLE CONTROL | ALKYLPHENOLS | E2 5 µg/L | E2 50 µg/L |
|---|---|---|---|---|---|
| **$V_v$ (bFCA) [a]** | Average | 0.36 | 0.40 | 0.18 | 0.55 |
| | Median | 0.27 | 0.21 | 0.14 | 0.28 |
| | Min-Max | 0.00-1.77 | 0.00-3.28 | 0.00-0.68 | 0.0-4.06 |
| **$V_V$ (aFCA) [b]** | Average | 0.15 | 0.00 | 0.07 | 1.49 |
| | Median | 0.00 | 0.04 | 0.000 | 0.92 |
| | Min-Max | 0.00-2.76 | 0.00-3.55 | 0.00-1.34 | 0.00-9.12 |
| **$V_v$ (eFCA) [c]** | Average | 0.01 | 0.00 | 0.00 | 0.29 |
| | Median | 0.00 | 0.00 | 0.000 | 0.00 |
| | Min-Max | 0.00-0.22 | 0.00-0.04 | 0.00-0.00 | 0.00-4.79 |
| **$V_v$ (Pre-FCA) [d]** | Average | 0.02 | 0.22 | 0.03 | 0.62 |
| | Median | 0.00 | 0.05 | 0.017 | 0.38 |
| | Min-Max | 0.00-0.24 | 0.00-0.78 | 0.00-0.45 | 0.00-5.76 |
| **$V_V$ (Total FCA) [e]** | Average | 0.50 | 0.40 | 0.26 | 2.33 |
| | Median | 0.30 | 0.30 | 0.177 | 1.72 |
| | Min-Max | 0.00-4.44 | 0.00-3.55 | 0.00-1.46 | 0.02-13.08 |
| **$V_v$ (TAP) [f]** | Average | 0.52 | 0.62 | 0.29 | 2.95 |
| | Median | 0.35 | 0.37 | 0.195 | 2.47 |
| | Min-Max | 0.00-4.44 | 0.00-3.55 | 0.02-1.46 | 0.16-14.21 |

[a] Relative volume ($V_V$) of basophilic foci of cellular alteration (FCA); [b] $V_V$ of amphophilic FCA; [c] $V_V$ of eosinophilic FCA; [d] $V_V$ of Pre-FCA; [e] $V_V$ of FCA; [f] $V_V$ sum of all FCA types; [g] $V_V$ of Total Altered Parenchyma ($V_V$ sum of all FCA types and Pre-FCA)

**Table 5- Significance of the difference (p-values) between groups, under the Mann-Whitney U test, considering the relative volumes of foci of cellular alteration (FCA) and Pre-FCA.**

| | VEHICLE CONTROL | | | | | |
|---|---|---|---|---|---|---|
| **ALKYLPHENOLS** | $V_V$ (bFCA) [a] | 0,12 | | | | |
| | $V_V$ (aFCA) [b] | **0.01** | | | | |
| | $V_V$ (eFCA) [c] | 0.94 | | | | |
| | $V_V$ (Pre-FCA) [d] | **< 0.0002** | | | | |
| | $V_V$ (Total FCA) [f] | 0.82 | | | | |
| | $V_V$ (TAP) [g] | 0.319 | **ALKYLPHENOLS** | | | |
| **E2 5 µg/L** | $V_V$ (bFCA) [a] | **0.00045** | $V_V$ (bFCA) [a] | 0.12 | | |
| | $V_V$ (aFCA) [b] | 0.07 | $V_V$ (aFCA) [b] | **< 0.0002** | | |
| | $V_V$ (eFCA) [c] | 0.22 | $V_V$ (eFCA) [c] | 0.23 | | |
| | $V_V$ (Pre-FCA) [d] | 0.06 | $V_V$ (Pre-FCA) [d] | **0.02** | | |
| | $V_V$ (Total FCA) [f] | **0.00034** | $V_V$ (Total FCA) [f] | **0.0016** | | |
| | $V_V$ (TAP) [g] | **0.00135** | $V_V$ (TAP) [g] | **< 0.0002** | **E2 5 µg/L** | |
| **E2 50 µg/L** | $V_V$ (bFCA) [a] | 0.94 | $V_V$ (bFCA) [a] | 0.15 | $V_V$ (bFCA) [a] | **0.0059** |
| | $V_V$ (aFCA) [b] | **< 0.0002** | $V_V$ (aFCA) [b] | **< 0.0002** | $V_V$ (aFCA) [b] | **< 0.0002** |
| | $V_V$ (eFCA) [c] | **< 0.0002** | $V_V$ (eFCA) [c] | **< 0.0002** | $V_V$ (eFCA) [c] | **< 0.0002** |
| | $V_V$ (Pre-FCA) [d] | **< 0.0002** | $V_V$ (Pre-FCA) [d] | **< 0.0002** | $V_V$ (Pre-FCA) [d] | **< 0.0002** |
| | $V_V$ (Total FCA) [f] | **< 0.0002** | $V_V$ (Total FCA) [f] | **< 0.0002** | $V_V$ (Total FCA) [f] | **< 0.0002** |
| | $V_V$ (TAP) [g] | **< 0.0002** | $V_V$ (TAP) [g] | **< 0.0002** | $V_V$ (TAP) [g] | **< 0.0002** |

[a] Relative volume ($V_V$) of basophilic foci of cellular alteration (FCA); [b] $V_V$ of amphophilic FCA; [c] $V_V$ of eosinophilic FCA; [d] $V_V$ of Pre-FCA; [e] $V_V$ of FCA; [f] $V_V$ sum of all FCA types; [g] $V_V$ of Total Altered Parenchyma ($V_V$ sum of all FCA types and Pre-FCA)

**Table 6 – Descriptive summary of the statistics of the relative volumes ($V_V$).***

| Volumes | Observations |
|---|---|
| **$V_v$ (bFCA)** | Vehicle Control greater than E2 5 µg/L<br>E2 5 µg/L smaller than E2 50 µg/L |
| **$V_v$ (aFCA)** | Vehicle control equal to E2 5 µg/L |
| **$V_v$ (eFCA)** | E2 50 µg/L higher than all groups<br>Vehicle control equal to Alkylphenols and E2 5 µg/L<br>Alkylphenols equal to E2 5 µg/L |
| **$V_v$ (Pre-FCA)** | Vehicle Control equal to E2 5 µg/L |
| **$V_v$ (Total FCA)** | Vehicle Control equal to Alkylphenols |
| **$V_v$ (TAP)** | Vehicle Control equal to Alkylphenols |

* The results of non-parametric *versus* parametric analyses produce similar deductions. See Tables 4 and 5 for the data and also for the elucidation of the nomenclature.

Overall, and considering the volume densities, it seems to be a concordance with the previous results regarding the prevalence of the lesions, since E2 50 µg/L differ from the other groups in all volumes, except in $V_v$ (bFCA). The Alkylphenols group presented equal volumes to Vehicle Control group, except in $V_v$ (aFCA) and $V_v$ (Pre-FCA). The Vehicle Control and E2 5 µg/L groups differed in three volume — $V_v$ (aFCA), $V_v$ (eFCA) an $V_v$ (Pre-FCA) — and were equal in the other three. The Alkylphenols differ from E2 50 µg/L group, except in $V_v$ (bFCA). The only groups that presented equal values of $V_v$ (Total FCA) and $V_v$ (TAP) were Alkylphenols and Vehicle Control.

## NORMAL PARENCHYMA: WITHOUT LESIONS *VS* WITH LESIONS

The volumes of the hepatocyte nucleus and of the all cell, along with the coefficient of variation, for cells in the parenchyma of animal with not lesions at all and in fish displaying lesions are summarized in Tables 7 and 8. The Blank Control group presented in all the parameters the lowest values, regarding the averages, and it also differed from the other four groups with lesions, in all the parameters. The four groups with lesions do not differ from each other, but, overall, they seem to show less interindividual variability as noted by the CV of the three volumes. Irrespective of this, the $CV_n$ ($\nu$) were exactly equal in all the groups (i.e., within a fish, the nuclei varied in the same extent).

**Table 7 – Parenchyma descriptive statistical data from each experimental group, including blank control, regarding the liver cell body and nuclear volume-related parameters (fish, n = 100)**

| | | Blank Control | Vehicle Control | Alkylphenols | E2 5 µg/L | E2 50 µg/L |
|---|---|---|---|---|---|---|
| $\overline{v}_n$ (nucleus) [a] | Average | 55.5 | 71.6 | 71.3 | 72.8 | 70.8 |
| | Min- Max | 42.6-70.7 | 61.0-92.0 | 61.9-80.3 | 67.2-78.8 | 64.2-78.4 |
| | Coefficient of variation | 0.12 | 0.11 | 0.07 | 0.05 | 0.06 |
| $\overline{v}_n$ (hepatocyte) [b] | Average | 768,0 | 836.9 | 881.9 | 848.7 | 880.8 |
| | Min- Max | 527.5-940.2 | 679.0-998.4 | 775.0-972.5 | 700.1-975.7 | 754.5-1066.3 |
| | Coefficient of variation | 0.14 | 0.09 | 0.07 | 0.09 | 0.10 |
| $\overline{v}_v$ (nucleus) [c] | Average | 72.1 | 101.9 | 101.9 | 104.0 | 100.8 |
| | Min- Max | 60.5-81.8 | 83.0-122.2 | 91.1-120.0 | 87.7-119.2 | 93.0-110.2 |
| | Coefficient of variation | 0.08 | 0.12 | 0.09 | 0.08 | 0.05 |
| $CV_n$ (ν) [d] | Average | 0.5 | 0.6 | 0.7 | 0.7 | 0.7 |
| | Min- Max | 0.3-0.8 | 0.5-0.8 | 0.6-0.8 | 0.5-0.8 | 0.6-0.8 |
| | Coefficient of variation | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 |

[a] Number-weighted volume of the hepatocyte nucleus ($\mu m^3$)
[b] Number-weighted volume of the hepatocyte ($\mu m^3$)
[c] Volume -weighted volume of hepatocyte nucleus ($\mu m^3$)
[d] Coefficient of variation of the hepatocytes nucleus in the number-weighted distribution.

**Table 8 – Significance of the difference (p-values) between experimental groups, under the Newman-Keuls test, regarding the liver cell body and nuclear volume-related parameters in the normal parenchyma.**

| | BLANK CONTROL | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| VEHICLE CONTROL | $\bar{v}_n$ (nucleus) [a] | **< 0.0002** | | | | | | |
| | $\bar{v}_n$ (hepatocyte) [b] | **0,0102** | | | | | | |
| | $\bar{v}_v$ (nucleus) [c] | **< 0.0002** | | | | | | |
| | CVn (V) [d] | **< 0.001** | VEHICLE CONTROL | | | | | |
| ALKYLPHENOLS | $\bar{v}_n$ (nucleus) [a] | **< 0.0002** | $\bar{v}_n$ (nucleus) [a] | 0,8737 | | | | |
| | $\bar{v}_n$ (hepatocyte) [b] | **< 0.001** | $\bar{v}_n$ (hepatocyte) [b] | 0,3225 | | | | |
| | $\bar{v}_v$ (nucleus) [c] | **< 0.0002** | $\bar{v}_v$ (nucleus) [c] | 0,8534 | | | | |
| | CVn (V) [d] | **<0.01** | CVn (V) [d] | 0,9737 | ALKYLPHENOLS | | | |
| E2 5 µg/L | $\bar{v}_n$ (nucleus) [a] | **< 0.0002** | $\bar{v}_n$ (nucleus) [a] | 0,5044 | $\bar{v}_n$ (nucleus) [a] | 0,6856 | | |
| | $\bar{v}_n$ (hepatocyte) [b] | **< 0.001** | $\bar{v}_n$ (hepatocyte) [b] | 0,6564 | $\bar{v}_n$ (hepatocyte) [b] | 0,4173 | | |
| | $\bar{v}_v$ (nucleus) [c] | **< 0.0002** | $\bar{v}_v$ (nucleus) [c] | 0,7005 | $\bar{v}_v$ (nucleus) [c] | 0,5367 | | |
| | CVn (V) [d] | **< 0.01** | CVn (V) [d] | 0,9781 | CVn (V) [d] | 0,8214 | E2 5 µg/L | |
| E2 50 µg/L | $\bar{v}_n$ (nucleus) [a] | **< 0.0002** | $\bar{v}_n$ (nucleus) [a] | 0,8980 | $\bar{v}_n$ (nucleus) [a] | 0,7781 | $\bar{v}_n$ (nucleus) [a] | 0,6828 |
| | $\bar{v}_n$ (hepatocyte) [b] | **< 0.001** | $\bar{v}_n$ (hepatocyte) [b] | 0,2221 | $\bar{v}_n$ (hepatocyte) [b] | 0,9655 | $\bar{v}_n$ (hepatocyte) [b] | 0,2243 |
| | $\bar{v}_v$ (nucleus) [c] | **< 0.0002** | $\bar{v}_v$ (nucleus) [c] | 0,7030 | $\bar{v}_v$ (nucleus) [c] | 0,8375 | $\bar{v}_v$ (nucleus) [c] | 0,6358 |
| | CVn (V) [d] | **< 0.01** | CVn (V) [d] | 0,8508 | CVn (V) [d] | 0,9692 | CVn (V) [d] | 0,9905 |

[a] Number-weighted volume of the hepatocyte nucleus
[b] Number-weighted volume of the hepatocyte
[c] Volume -weighted volume of hepatocyte nucleus
[d] Coefficient of variation of the hepatocytes nucleus in the number-weighted distribution

## ALTERED PARENCHYMA: VOLUME PARAMETERS OF bFCA AND aFCA

The volumes of the hepatocyte nucleus and of the all cell, along with the coefficient of variation of the nuclear volume, within the bFCA, are summarized in Tables 9 and 10. From these data, we found that relative to bFCA, the $\bar{v}_n$ (nucleus) and the $\bar{v}_v$ (nucleus), E2 50 µg/L achieved the highest value, contrary to E2 5 µg/L, that presented the lowest. In these two parameters, all the groups were statistically different from E2 50 µg/L.

Concerning $\bar{v}_n$ (hepatocyte), we observed that the greatest volume is achieved in the Alkylphenols group, and that the lowest belongs, again, to the E2 5 µg/L group. For this volume, both the Alkylphenols and Vehicle Control groups were statistically different from the E2 5 µg/L exposure.

As to the $CV_n$ ($\nu$), the greater variability respecting the nucleus belongs to E2 50 µg/L group, being statistically different from the others; that even presented equal values.

Looking to the Ratio nucleus/cell (%), animals under E2 50 µg/L achieved the highest value, while the Vehicle Control group presented the lowest. The latter and the Alkylphenols groups were statistically different from both E2 50 µg/L and E2 5 µg/L, being these last two also different between themselves.

As to the $V_V$ (sinusoid, bFCA), the mean values were quite alike among groups. On the contrary, the $V_V$ (nuclei, bFCA) revealed significant differences among groups, with a trend for higher values in the groups exposed to estrogenic stimuli. Indeed, the E2 5 µg/L achieved the highest value, differing from those of the Alkylphenols and the Vehicle Control groups, with the E2 50 µg/L fish also differing from those of Vehicle Control.

Observing the results from aFCA described in Table 10, we verified that regarding the $\bar{v}_n$ (nucleus) and the $\bar{v}_v$ (nucleus) the E2 50 µg/L tended to promote the higher values and the E2 5 µg/L the lowest ones. Respecting the $\bar{v}_v$ (nucleus), the E2 5 µg/L and E2 50 µg/L conditions are statistically different.

Concerning the $\bar{v}_n$ (hepatocyte), we observed that the most elevated volume was seen in Alkylphenols group and the lowest belongs again in E2 5 µg/L group; also similar to bFCA. In this case, Vehicle Control and Alkylphenols differ from E2 5 µg/L group.

As to the $CV_n$ ($\nu$) — representing the volume variability in the number-weighted distribution —, the E2 50 µg/L group showed the highest one, being statistically differ-

ent from the other groups (that presented similar values). In general, the $CV_n$ ($\nu$) was very high, with values always over 60%.

Like for the bFCA, in the aFCA the E2 50 µg/L achieved the highest value in the Ratio nucleus/cell (%) — that is the same to say the $V_V$ (nucleus, hepatocyte) —, while the Alkylphenols group presented the lowest one. Note that the E2 50 µg/L and E2 5 µg/L groups differ from the Alkylphenols; the Control had an intermediate value.

Looking to the volumetric densities, in $V_V$ (sinusoid, aFCA) the groups were not statically different from each other. Both the E2 5 µg/L and E2 50 µg/L groups presented the greater values of $V_V$ (nucleus, aFCA) and differed significantly from the Vehicle Control, being Alkylphenols group placed in between.

It was not possible to accomplish a statistical analysis of eFCA, since the number of positive cases was only sufficient in E2 50 µg/L; which makes it impossible to perform comparisons between all groups.

**Table 9- Descriptive statistical data from basophilic foci of cellular alteration (bFCA), regarding the liver cell body and nuclear volume-related parameters and sinusoid and nucleus relative volumes (fish, n = 20).**

| | | Vehicle Control | Alkylphenols | E2 5 µg/L | E2 50 µg/L |
|---|---|---|---|---|---|
| $\overline{v}_n$ (nucleus) [a] | Average | 97.7* | 100.8* | 95.9* | 108.7 |
| | SD | 7.2 | 6.9 | 11.9 | 9.08 |
| $\overline{v}_n$ (hepatocyte) [b] | Average | 886.8** | 898.7** | 78.1 | 845.0 |
| | SD | 85.8 | 75.7 | 82.1 | 84.1 |
| $\overline{v}_v$ (nucleus) [c] | Average | 139.6* | 143.1* | 136.4* | 162.6 |
| | SD | 11.5 | 11.5 | 18.7 | 13.6 |
| $CV_n$ (v) [d] | Average | 0.7* | 0.7* | 0.7* | 0.7 |
| | SD | 0.1 | 0.0 | 0.1 | 0.1 |
| Ratio nucleus/cell (%) | Average | 11.1*/** | 11.3*/** | 12.1* | 12.9** |
| | SD | 0.0 | 0.0 | 0.0 | 0.0 |
| $V_V$ (sinusoid) (%) [e] | Average | 13.2 | 14.1 | 15.1 | 13.5 |
| | SD | 0.0 | 0.0 | 0.0 | 0.0 |
| $V_V$ (nucleus) (%) [f] | Average | 6.3 | 6.8**** | 8.0***/**** | 7.4*** |
| | SD | 0.0 | 0.0 | 0.0 | 0.0 |

SD- Standard Deviation

[a] Number-weighted volume of the hepatocyte nucleus (µm3)
[b] Number-weighted volume of the hepatocyte (µm3)
[c] Volume -weighted volume of hepatocyte nucleus (µm3)
[d] Coefficient of variation of the hepatocytes nucleus in the number-weighted distribution
[e-] Relative volume (sinusoid, reference space)
[f-] Relative volume (nucleus, reference space)

* statistically different from E2 50 µg/L group; p <0.01 in $\overline{v}_n$ (nucleus); p <0.001 in $\overline{v}_n$ (hepatocyte); p <0.05 in $CV_n$ (v) and ratio nucleus / cell
** statistically different from E2 5 µg/L group; p <0.01 in $\overline{v}_n$ (hepatocyte); p <0.05 in the ratio nucleus / cell
*** statistically different from Vehicle Control (p <0.001)
**** statistically different between themselves (p <0.001)

**Table 10- Descriptive statistical data from amphophilic foci of cellular alteration (aFCA), regarding the liver cell body and nuclear volume-related parameters and sinusoid and nucleus relative volumes, (fish, n = 20).**

| | | Vehicle Control | Alkylphenols | E2 5 µg/L | E2 50 µg/L |
|---|---|---|---|---|---|
| $\overline{v}_n$ (nucleus) [a] | Average | 105.8 | 101.3 | 98,6 | 107.8 |
| | SD | 15.1 | 10.5 | 11.5 | 10.2 |
| $\overline{v}_n$ (hepatocyte) [b] | Average | 901.4** | 926.7** | 806.7 | 856.1 |
| | SD | 92.7 | 70.0 | 86.6 | 96.7 |
| $\overline{v}_v$ (nucleus) [c] | Average | 150.9 | 145.4 | 138.3***** | 162.8***** |
| | SD | 23.0 | 16.1 | 11.8 | 17.7 |
| $CV_n$ (ν) [d] | Average | 0.7* | 0,7* | 0.6* | 0.7 |
| | SD | 0.1 | 0.0 | 0.1 | 0.1 |
| Ratio nucleus/cell (%) | Average | 11.7 | 11.0 | 12.3**** | 12.7**** |
| | SD | 0.0 | 0.0 | 0.0 | 0.0 |
| $V_V$ (sinusoid) (%) [e] | Average | 13.1 | 14.8 | 13.1 | 13.3 |
| | SD | 0.0 | 0.0 | 0.0 | 0.0 |
| $V_V$ (nucleus) (%) [f] | Average | 6.1 | 6.7 | 7.1*** | 7.5*** |
| | SD | 0.0 | 0.0 | 0.0 | 0.0 |

SD- Standard Deviation

[a] Number-weighted volume of the hepatocyte nucleus (µm3)
[b] Number-weighted volume of the hepatocyte (µm3)
[c] Volume -weighted volume of hepatocyte nucleus (µm3)
[d] Coefficient of variation of the hepatocytes nucleus in the number-weighted distribution
[e-] Relative volume (sinusoid, reference space)
[f-] Relative volume (nucleus, reference space)

* statistically different from E2 50 µg/L group; p <0.01 in $\overline{v}_n$ (nucleus); p <0.001 in $\overline{v}_n$ (hepatocyte); p <0.05 in $CV_n$ (ν) and ratio nucleus / cell
** statistically different from E2 5 µg/L group; p <0.01 in $\overline{v}_n$ (hepatocyte); p <0.05 in the ratio nucleus / cell
*** statistically different from Vehicle Control (p <0.001)
**** statistically different between themselves (p <0.001

## HISTOCHEMISTRY

A summary of the data is offered in Table 11. Overall, the Sirius red (Figures 5 and 6) and the PAS (Figures 7, 8 and 9) stainings evidenced that the parenchyma in the FCA could be made histochemically distinguishable from the remaining liver *muralium*, and that there was diversity as to the hepatocyte glycogen content for FCA of same type. The Perls' staining (Figure 4) did not evidenced the presence of ferric salts.

**Table 11- Histochemistry expected outcomes and results.**

| Stain | Expected outcome | Result |
|---|---|---|
| **Perls'** | Ferric salts: blue<br>Nuclei: pink | Negative: absence of ferric salts. |
| **Sirius red** | Collagen: red<br>Nuclei: brown | The delicate perisinusoidal stroma did not differ in thickness within the FCA of the diverse types and also in the surrounding normal parenchyma; except in the case of one adenoma, where we noted a slight increase. |
| **PAS** | Glycogen: red/purple<br>Nuclei: blue | Heterogeneity between FCA of the same type, observing in the majority of the cases less glycogen within the FCA hepatocytes, when comparing with the surrounding parenchyma. |

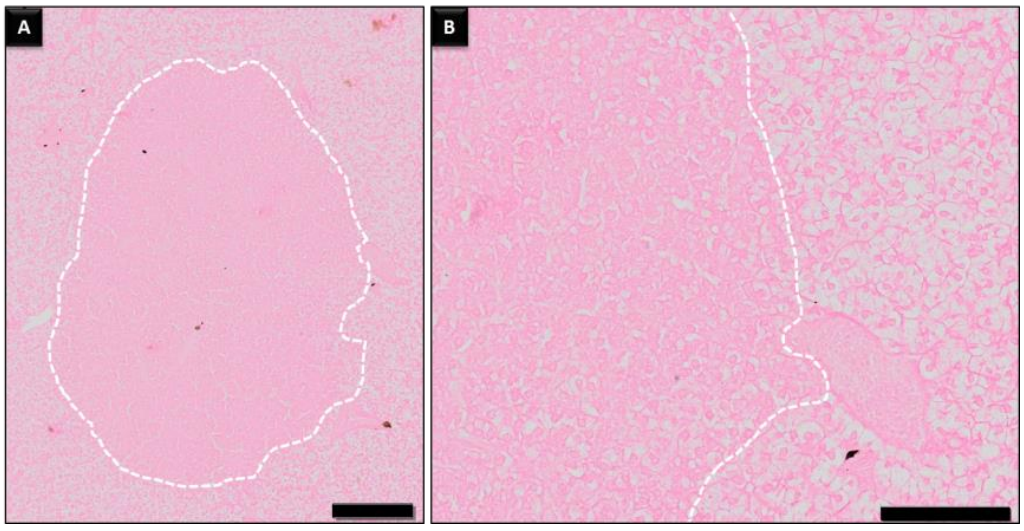


Figure 4 Perls' staining. The FCA (delimited in A and detailed in B) marked negatively for ferric salts. Bars: A- 200 µm; B- 100 µm.

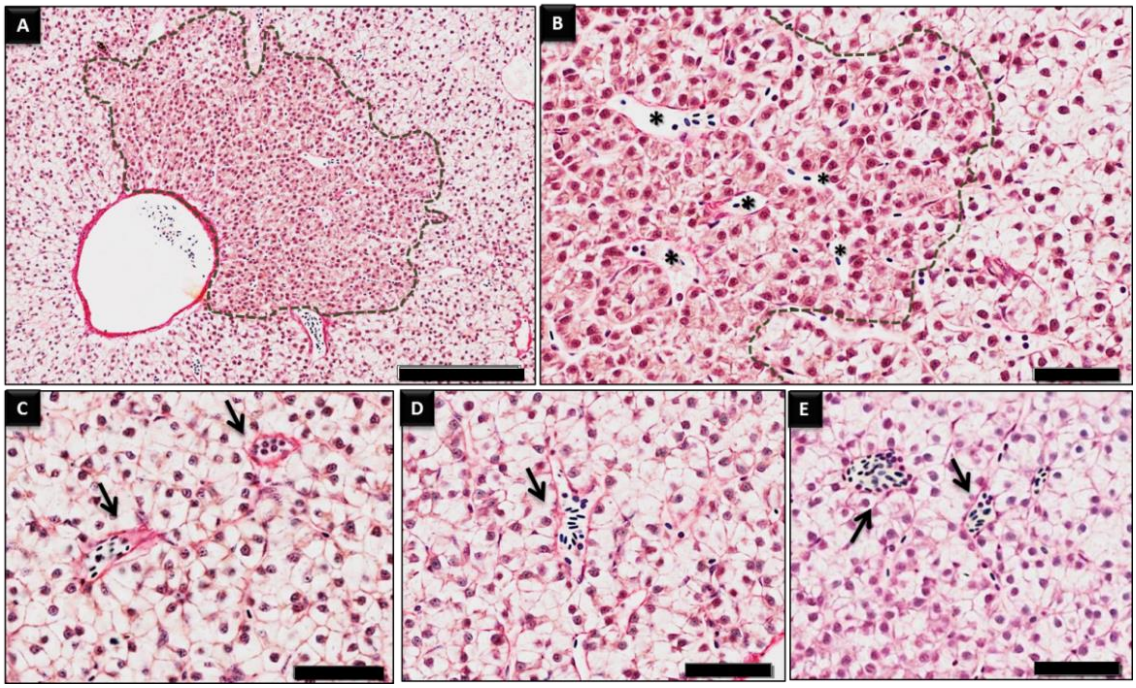


Figure 5 Sirius red staining. Perisinusoidal collagen stains lightly, with a reddish tone. There are no differences between walls of the smaller blood vessels (*) within the FCA (delimited in A and detailed in B) and the ones (arrows) of the surrounding parenchyma (C-E). Bars: A- 200 µm; B to D- 50 µm.

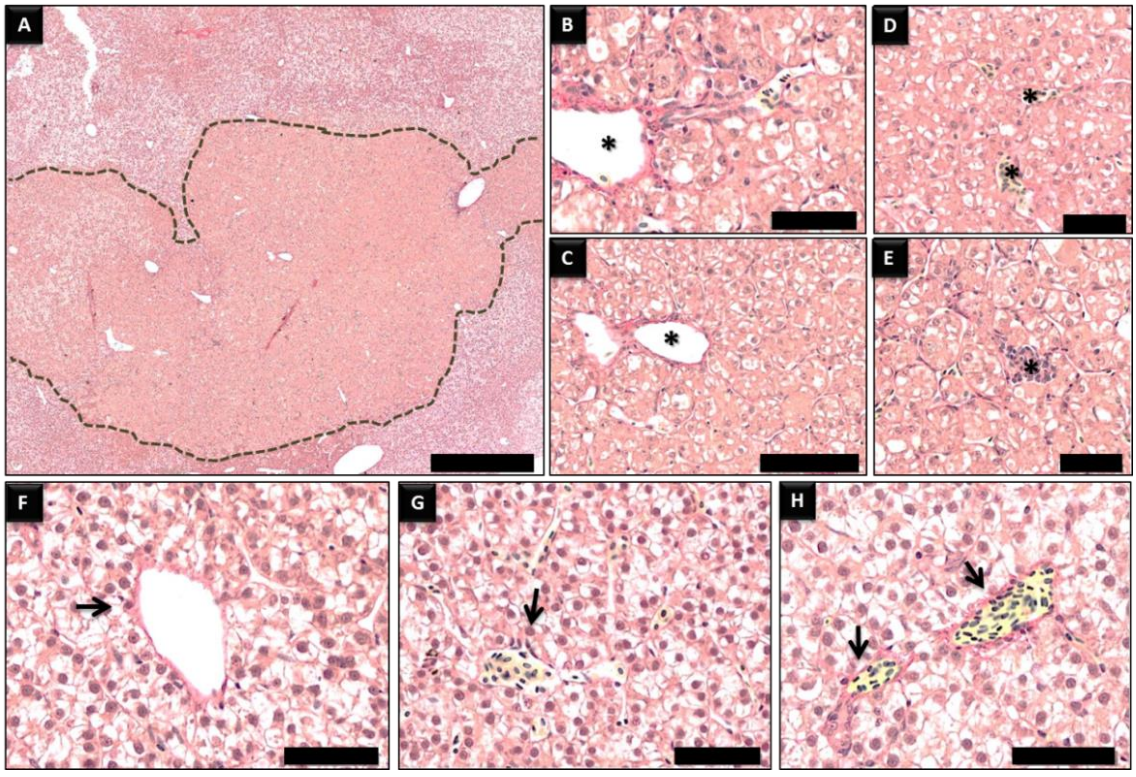


Figure 6 Sirius red staining. General overview of an adenoma (delimited in A), along with some details (B-E), and exemplificative images of the normal parenchyma nearby (F-I). The adenoma blood vessels (*) look slightly more thickened — regarding the collagen content of their wall and/or perisinusoidal space — than in those (arrows) of the surrounding parenchyma (F-I). Bars: A – 500 µm; B to H - 50 µm, except C- 100 µm.

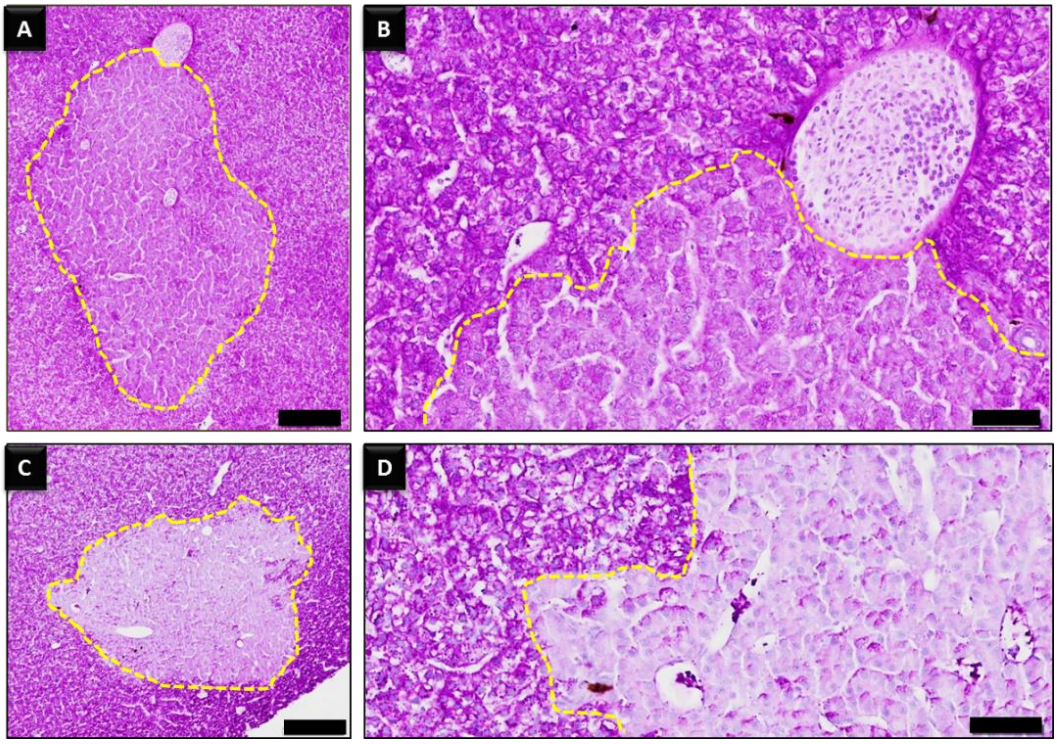


Figure 7 PAS Staining. Two amphophilic FCA (delimited in A and C and detailed in B and D) that present less glycogen within hepatocytes when comparing with the hepatocellular load in the surrounding parenchyma. Bars: A – 200 µm; B - 50 µm; C – 200 µm; D - 50 µm.

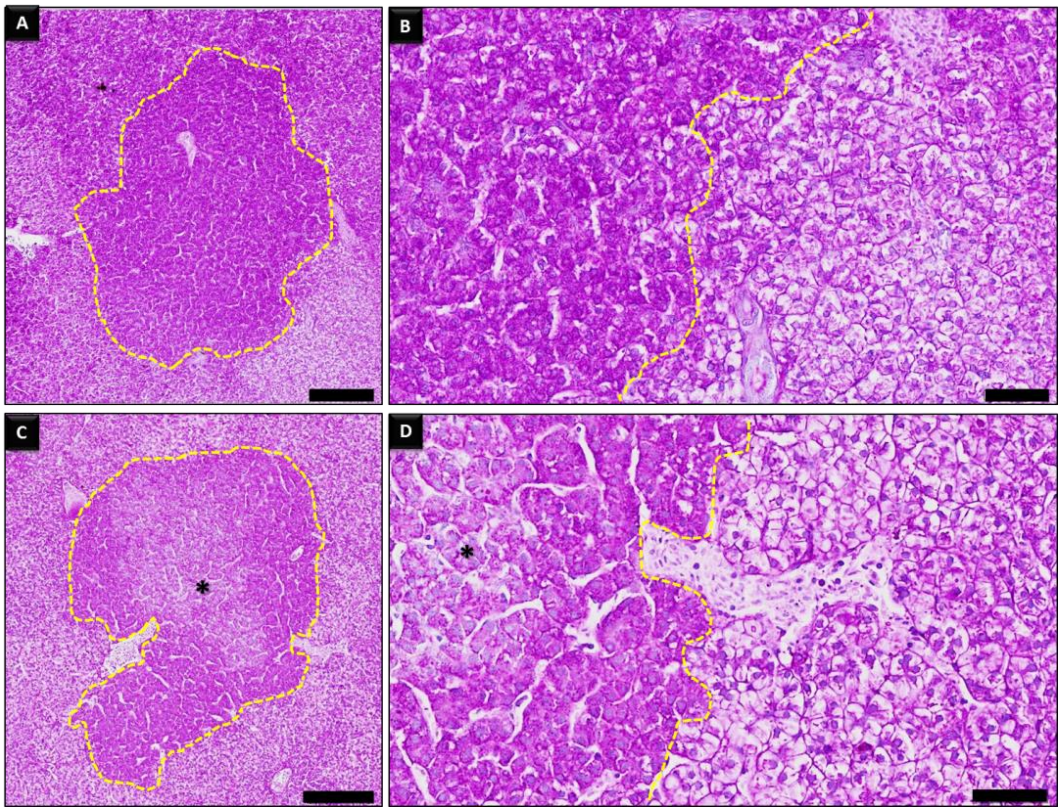


Figure 8 PAS Staining. Two amphophilic FCA (delimited in A and C and detailed in B and D) that present more glycogen compared with the surrounding parenchyma, contrary to the observed in Figure 8. In the amphophilic FCA in C, two staining compartments are seen simulta-

neously: one in the center of the FCA (*), staining somewhat more close to the surrounding parenchyma, and other, at the FCA border (matching to a more basophilic periphery in Hematoxylin and Eosin staining), presenting more glycogen than the surrounding parenchyma. Bars: A- 200 µm; B 50 µm; C- 200 µm; D- 50 µm.

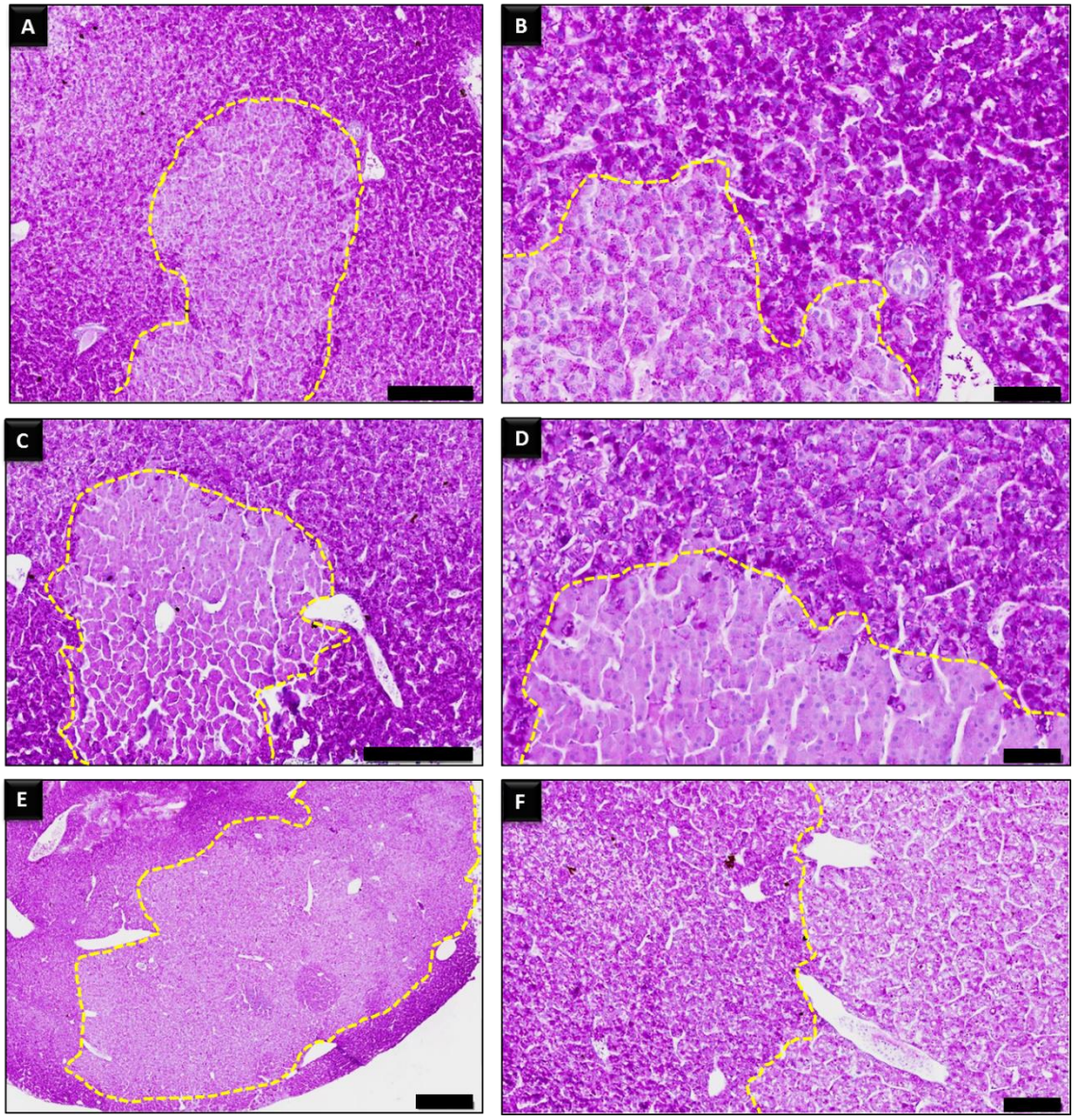


Figure 9 PAS Staining. The eosinophilic FCA at the top (delimited in A and detailed in B), the basophilic FCA in the middle (delimited in C and detailed in D), and the adenoma on the bottom (delimited in E and detailed in F), all present less glycogen compared with the adjacent normal parenchyma. Bars: A- 200 µm, B- 50 µm, C- 200 µm, D – 50 µm, E- 500 µm, F - 100 µm.

## IMMUNOHISTOCHEMISTRY

Regarding E-cadherin immunoreactivity (Figure 10), we observed a “negative pattern” (i.e., less staining intensity at the boundaries between individual hepatocytes) within all the types of FCA, when compared to the surrounding parenchyma.

Respecting CYP1A immunostaining, we noted heterogeneity among amphophilic FCA (Figure 11), with distinct patterns of immunoreactivity, going from very weak to strong signal. Eosinophilic FCA (Figure 12) also presented heterogeneity, but less pronounced than aFCA, with a slight to a moderated intensity. We detected the weaker signal in basophilic FCA, which seem to present a more linear pattern (Figure 13).

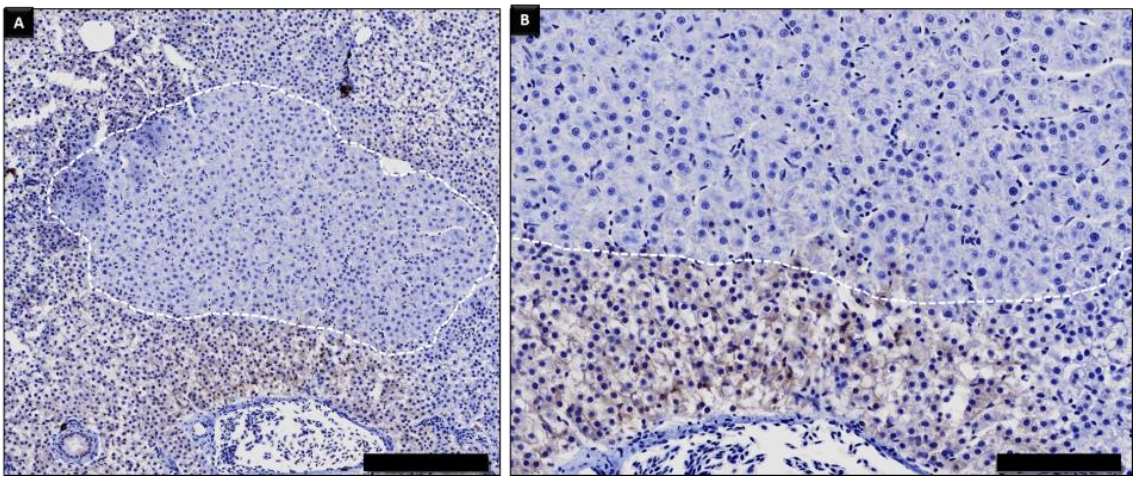


Figure 10 E-cadherin immunoreactivity in FCA (delimited in A and detailed in B). In the latter, it becomes evident the nearly absence of staining comparing with the surrounding parenchyma (specially, close to blood vessels); a feature apparently similar in all the FCA types. Bars: A- 200 µm; B- 100 µm.

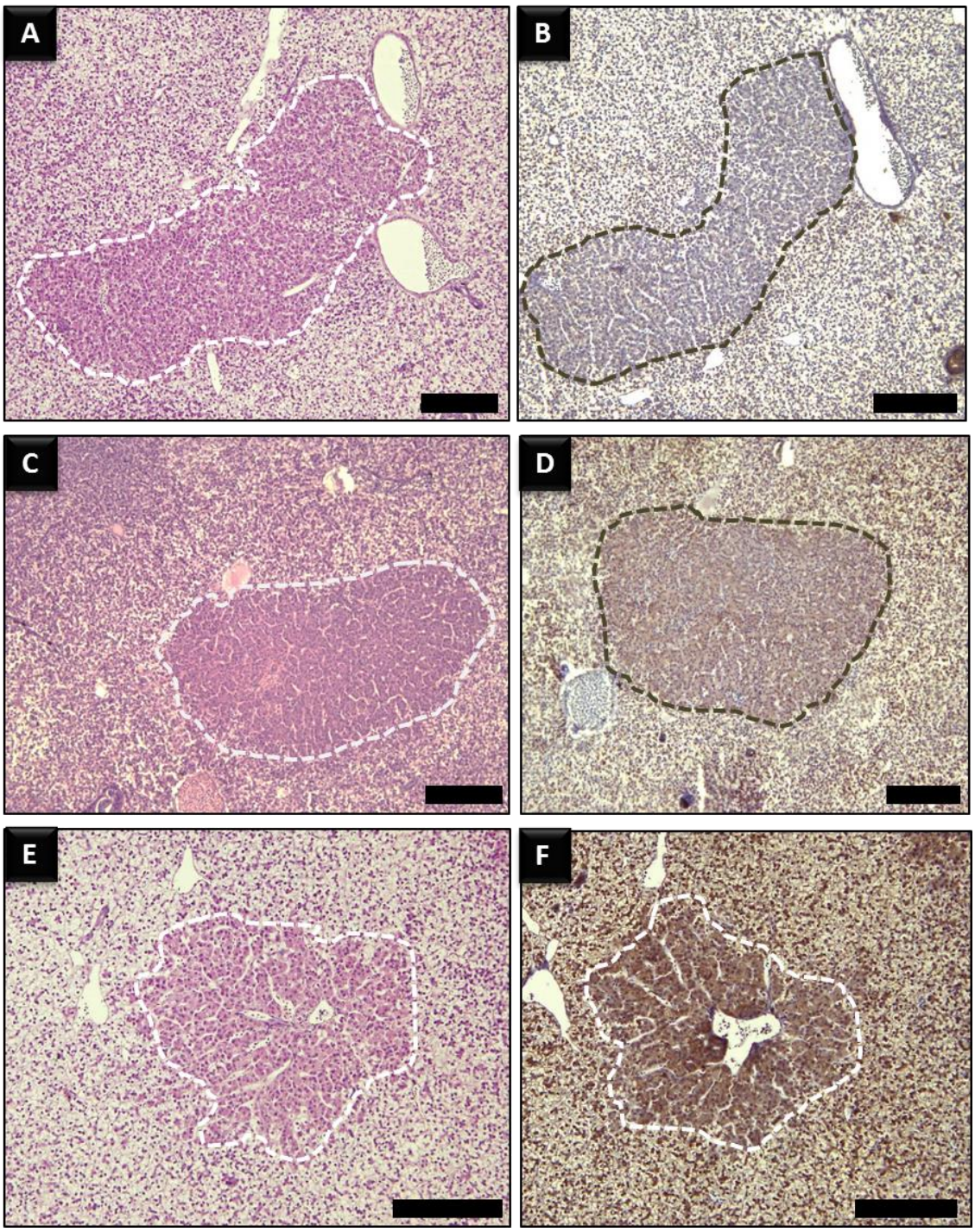


Figure 11 Hematoxylin and Eosin staining (delimited FCA in A,C and E) and CYP1A immunoreactivity (delimited FCA in B,D and F) in amphophilic FCA. There is heterogeneity between the depicted FCA, with distinct patterns of staining reactivity, going from very slight (B), to moderated (D), and even to very intense (F). Bars: A to F- 20 µm.

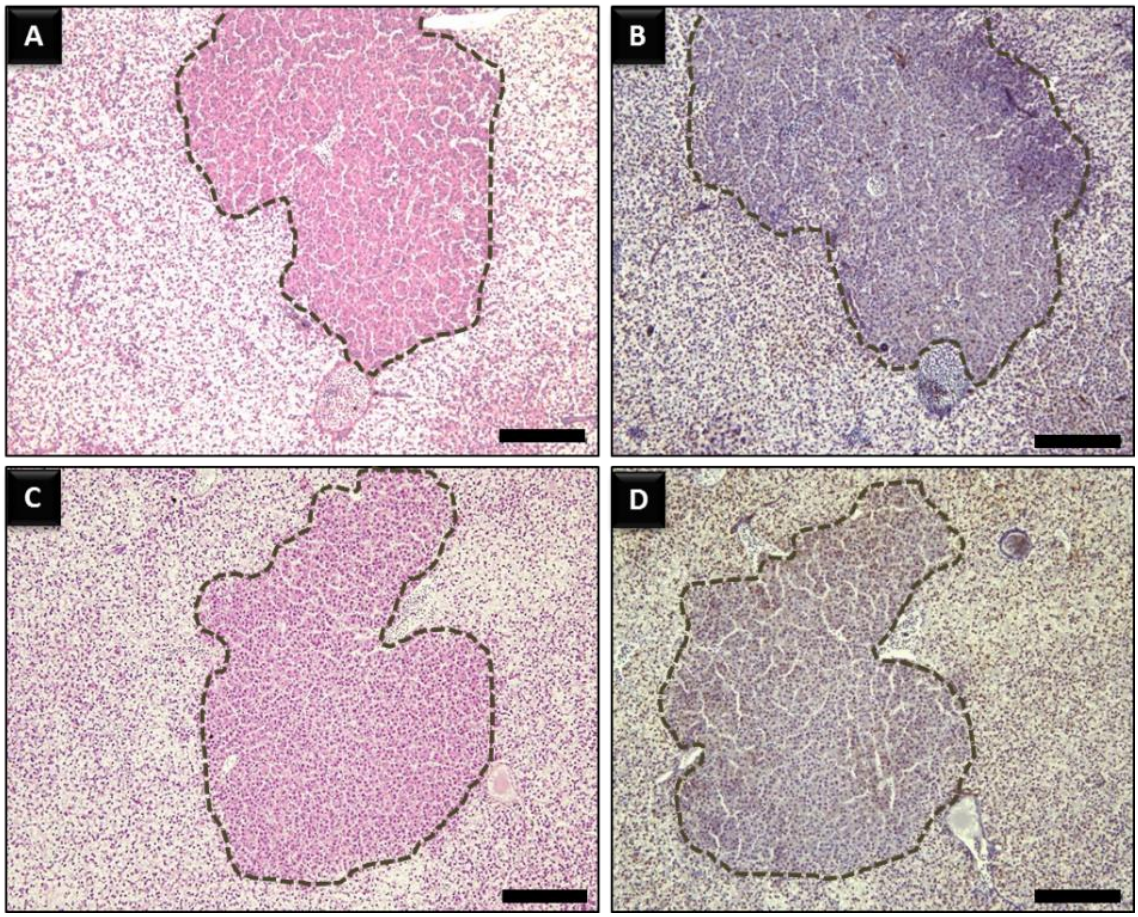


Figure 12 Hematoxylin and Eosin staining (delimited FCA in A, C and E) and CYP1A immunoreactivity (delimited FCA in B, D and F) in eosinophilic FCA. These type of FCA present heterogeneity, less pronounced than in the amphophilic FCA, with a slight (B) to moderate intensity (D). Bars: A to D- 20 µm.

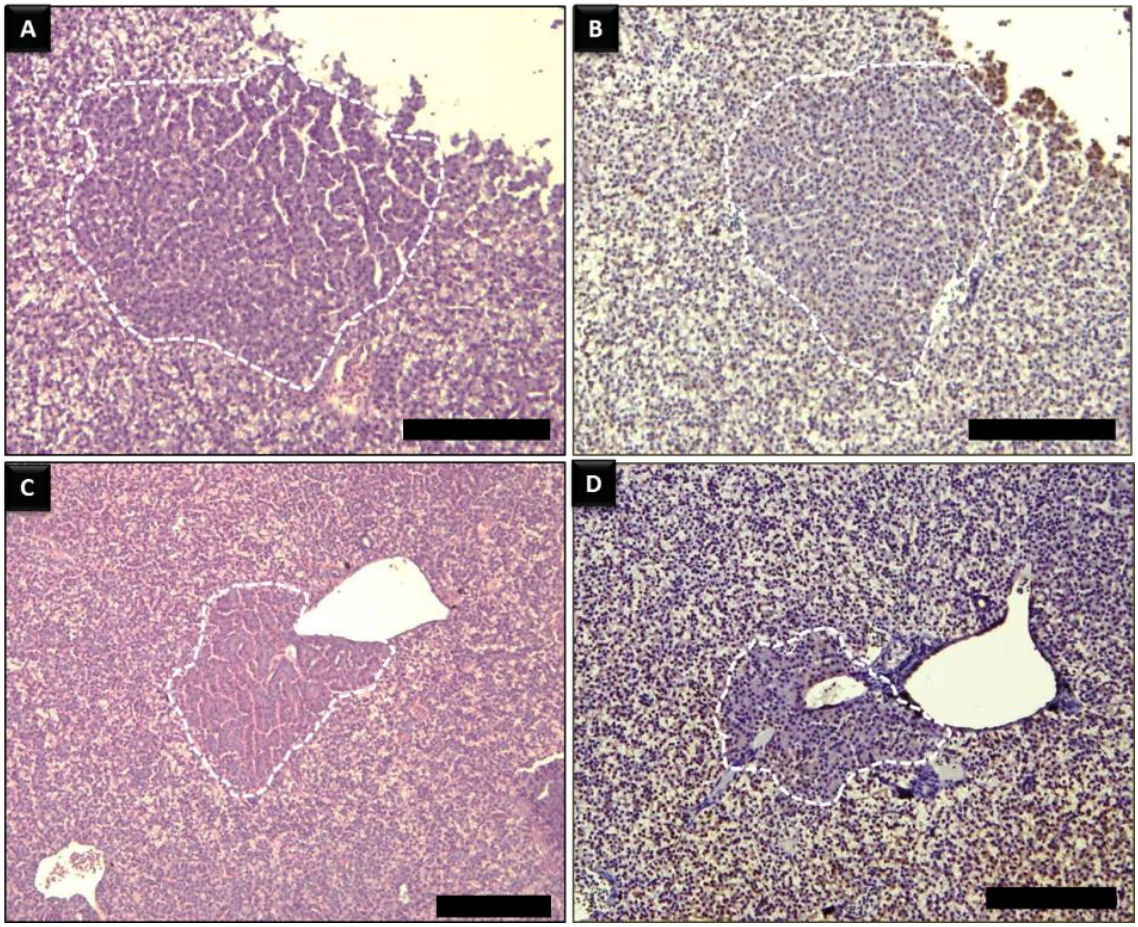


Figure 13 Hematoxylin and Eosin staining (delimited FCA in A and C) and CYP1A immunoreactivity (delimited FCA in B and D) in basophilic FCA. This type of FCA presents a weak signal when compared with the amphophilic and eosinophilic FCA. Bars: A to D- 20 µm.

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# DISCUSSION

# DISCUSSION

## OPTICAL MICROSCOPY *VS* DIGITAL MICROSCOPY

As Romer and Suster (2003) emphasized pathology professors have resorted to traditional glass slides and standard microscopes for many decades. However, they have faced some problems, because the slides are fragile and easy to break by careless students and difficult to duplicate, fading over time. Fortunately, e.g., according to Dee (2006), virtual microscopy has revolutionized the teaching of microscopic pathology, since multiple medical schools have implemented this novel technology. Summarizing, virtual microscopy is very attractive to professors because it nearly perfectly simulates the plan and zoom features of traditional microscopy, and presents in addiction numerous advantages like efficiency (adequate focus; proper condenser adjustment; easier viewing technique), accessibility (rapid access anywhere and anytime with a computer; one slide can be analyzed at the same time by several viewers), and, at last, versatility of computer-assisted education.

It seems that the only disadvantage that really stands out is the expensive costs for the acquisition and maintenance of equipment; despite in the experience of the Histology and Embryology Laboratory of the ICBAS he latter aspect is not particularly different from any other technically delicate equipment, like excellent microtomes and research microscopes (Eduardo Rocha, personal communication). Accordingly, Dee (2006) foresaw that costs would come down with innovation and competition among the companies. Besides that, the students have to learn how to use a traditional microscope, so the exclusive train with virtual microscopy is not recommended, despite students had rated the quality of images and navigation of the virtual microscope equal to or better than a real microscope (Harris et al, 2001).

Regarding the current routine diagnosis of pathological cases, several studies already used the virtual microscopy, and the results pointed that the information withdrawal of the imaging analysis is sufficient to make reliable diagnostic decisions and compose complex diagnostic reports, where the accuracy of virtual microscopy can match the glass slide microscopy (Gilbertson et al. 2006; Ficsor et al. 2008; Evered and Dudding 2011). Pathologists also highlight the reduction in the time of diagnosis and better image quality in frozen sections (Al-Janabi et al 2012). We cannot also forget that archived digital slides are a huge data warehouse containing valuable data for the future (Al-Janabi et al 2012).

As mentioned before, this work was divided in two phases, giving us the first one an overview concerning the lesions behavior towards the chemicals administration, which allowed us to plan the second one. In the end, we concluded that the FCA diagnosis based on virtual slides analysis was optimized, in speed, in commodity for the observer, and in our view (despite no statistics were involved) rising its sensitivity and specificity. In addition, the measurement tools provided by VS120-SL were definitely an added value.

From our experience with this study, the virtual slide scans and attached image analysis programs are clearly a valuable tool in this type of screening diagnosis and quantification. It should be noted that virtual microscopy has been introduced in comparative pathology basically in the last decade (Dee, 2006), and research in fishes using this technology is still very rare; studies such us ours support and will encourage a wider use of this approach.

## BASELINE RATE AND PROMOTION EFFECT

The liver is a key target organ in preclinical toxicity and oncogenicity assessment. Despite this fact, the importance of hepatic neoplastic lesions in animal models has been questioned concerning their predictive value, since humans seem to be more resistant to certain carcinogenic agents (Thoolen et al., 2012). Aside from the more or less predictive value, it seems consensual that the early detection and interpretation of proliferative and nonproliferative hepatic lesions can be relevant to explain how the toxicity to the liver is considered to be the second most frequent cause of drug failure (Thoolen et al., 2012).

Several studies have been describing over decades pre-neoplastic lesions in liver, initiated and/or promoted by the administration of chemicals, either in experiments or not. For example, Stentiford et al. (2003), also applying histopathological analyses, observed an elevated prevalence of FCA in the liver of flounder captured from the locals with highest levels of polycyclic aromatic hydrocarbons (PAH) contamination. Moreover, high prevalences of FCA in livers of fishes are well documented from polluted sites in the North American coast, in UK estuaries and also in the Baltic Sea (Fricke et al, 2012). In a totally different context, Tsuchishima et al (2013) verified the effects of ethanol on the formation of precancerous FCA in mice and suggested that chronic intake of alcohol produces extensive steatosis and accelerates the formation of preneoplastic lesions that further can lead to HCC. Very recently, using the alternative testing "in ovo carcinogenicity assay", Enzmanna et al. (2014), demonstrated that carcinogenic tobacco specific nitrosamine 4-(N-methyl-N-nitrosamine)-1-(3-pyridyl)-1-1-butanone

(NNK) induces FCA in fetal turkey liver. The primary exposure to MNNG succeeded herein to essentially induce the emergence of FCA only, being the initiator previously used in hepatocarcinogenesis protocols for rainbow trout (Bailey et al, 1996; Williams, 2012). Herein, estrogenic stimulation acted as a promoter, with E2 at the highest dose being more effective compared with the alkylphenols mixtures, as we hypothesized. With the promoters, it was observed an increase in both the number of affected fish and in the diversity of lesions types. It was clear that the group E2 50 µg/L stood out from the rest because of the higher frequencies; also, $V_V$ of the FCA were greater with the E2 50 µg/L. The cited group also stood out because of the distribution of the foci, being the one with the higher prevalence of aFCA and of the combination bFCA + aFCA, which contradicted the other groups. These facts seem not well in agreement with the most usual path pointed for FCA types evolution, presented in the Introduction, where it was stated that some authors point that bFCA is the preneoplastic stage that precedes the HCC. Moreover, at least for mammals, the amphophilic foci type is considered as a precursor of the basophilic phenotype, suggesting a morphological continuum between the two lesions. Based on this, we should have observed more eFCA and aFCA in the groups that the promoter effect was weaker (like Alkylphenols group), and more bFCA in the E2 50 µg/L group. However, we can also think that if the promoting effect is strong, more initial/intermediate foci could be enlarging and evolving to the more basophilic type. Indeed, the bFCA were significantly more frequent and had a greater $V_V$ in the E2 50 µg/L group than in any of the other groups. Whatever the true is, our data highlights once more the tricky nature of the FCA and the difficulty in interpreting its time evolution/regression.

The results from the group E2 5 µg/L, surprisingly, point for the potential paradoxical effects of estrogens — protector and promoter — since it was the one presenting the lowest values regarding the prevalence. This fact was also reported in the Introduction, when we cited studies that demonstrated the protective role of estrogens in different pathologies, such as HCC (Mendelsohn and Karas, 1999; Omoya et al, 2001; Shimizu et al., 2007; Kalra et al., 2008; Tian et al, 2012). This way, it seems that estrogens can have two opposite effects that can produce different outcomes in the environment, in which doses assume a crucial role — the higher the dose, the higher the probability of lesions promotion. Enzmann et al. (2013), using diethylnitrosamine (DEN) in turkey eggs, assessed FCA and concluded that its induction was clearly dose-dependent. Those authors even suggested that although FCA could be used to determine thresholds of effects of both genotoxic and non-genotoxic carcinogens, they additionally mentioned that it has been shown that thresholds for the induction of FCA and of tumors may be clearly different. The same reasoning could be applied to the pro-

moters, helping to explain not only dose-dependent effects but also cases of opposite effects of lower *vs* higher doses of a chemical. This apparent duality can be associated with the concept of hormesis: term used by toxicologists to describe a dose-response phenomenon in which an environmental agent that produces harmful biological effects at moderate to high doses may produce beneficial effects at low doses. So, in the presence of a low dose of a toxicant, the cell response can be considered an adaptive process to compensate an initial disruption in homeostasis (Calabrese, 2002; Mattson, 2008). Our results seem to favor this hypothesis under the studied experimental context, as in the Strom et al (2011) study, where they describe how the estrogens effects on stroke can be divergent, since hormone therapy can be neuroprotective, while, on the contrary, can increase stroke risk and ischemic lesions. The authors also referred that the estrogens protective potential can include multifactorial mechanisms, like apoptosis and inflammation decrease, beneficial vascular effects and growth factor modulation. Strom et al (2011) concluded that there is a necessity to assess the biological relevance of a wide range of doses, by serum hormone measurements and subsequent comparison with the intended biological situation. As stated by Vandenberg et al (2012), it is possible that "the dose makes the poison" dogma is indeed right, since estrogens can have effects at lower doses that are not predicted by effects at higher doses.

The Alkylphenols and Control Vehicle groups presented similar results concerning the prevalence and extension, even in the $V_V$ (Total FCA) and $V_V$ (TAP), however, the alkylphenols mixture seem to have a slightly more powerful effect, having a highest prevalence of aFCA and Pre-FCA (also exceeding in extension comparing with the Control Vehicle group) and being the only one, besides the E2 50 µg/L group, to present the "triad" bFCA + aFCA + eFCA. However, it seems that the alkylphenols used are not a potential carcinogenic promoter in our model. Consequently, facing the high nominal dosage used herein, it seems unlikely that, per se, environmental pollution by alkylphenols would pose a risk for increased carcinogenesis, at least for fish sensible as brown trout. By other hand, the estrogenic chemicals can sum their effects when in mixtures, as shown in vivo and in vitro, which means that collectively, they may represent a significant environmental risk, even when each compound is present at low-effect concentrations (Brian et al., 2007; Evans et al., 2012). So, notwithstanding those chemicals maybe assume as of low dangerousness, we cannot rule out the possibility of risks at long time, with chronic effects, thus the relevance of monitoring.

Despite the alkylphenols mixture has a lowest estrogenic potential then the E2 5 µg/L, it was clear that it caused more damaging effects (at least in view of the parameters considered in this study), probably by acting in other cellular pathways, independ-

ent of ERs; as discussed below. Note that E2 is a natural ligand and that alkylphenols are artificial ligands that bind indeed to ERs with estradiol, but are liable to cause several other toxic effects, especially in chronic high concentrations. Molecules from the alkylphenol family, like the 4-nonylphenol (NP) or the 4-tert-octylphenol (OP), are used in various industrialized processes and released in the environment, being common aquatic pollutants. Eventually they become persistent pollutants that are poorly eliminated by liver detoxification enzymes in mammals and that can affect the cells (Ajj et al, 2013). In fact, alkylphenols were detected in the urine of 95% of a human test population from the United States and, despite controversy, have been associated to health problems like reduction in fertility and meiotic disruptions (Calafat et al, 2005) Additionally, it has been reported that alkylphenols are toxic to animals, plants and microorganisms, including rainbow trout, where, elaborate tests were performed, for instance using primary culture of hepatocytes (Bhattacharya et al, 2008; Tollefsen et al, 2008).

Alkylphenols are considered endocrine disrupting compounds eventually enable to promote the progression of estrogen-dependent cancers and cause interference with reproductive functions and normal developmental of fish (Harris et al, 2000; Kayama et al, 2003; Soares et al, 2008; Tollefsen et al, 2008). The explanation to this is found in the experiments that indicate alkylphenols capacity to mimic estrogen mitogenic signaling, in mouse through the binding affinity to17β-estradiol (E2), in the range of 0.1% for the nuclear receptor ERa66 and of 50% for the transmembrane g-protein coupled estrogen receptor (GPER) (Ajj et al, 2013). In 1938, it was published the first evidence that alkylphenols could be oestrogenic, but only in 1991 it was observed that nonylphenol was capable of initiating proliferation in breast tumor cells as if oestrogens were present (Soares et al, 2008). Presently, it seems clear that alkylphenolic compounds may play a role in breast cancer incidence (Darbre, 2006; Villeneuve et al, 2010). Actually it is hypothesized that alkylphenols increase the probability of acquiring a mutation in oncogenes or tumor suppressor genes, either by interfering with the metabolism of endogenous steroid hormones or by becoming mutagenic upon sulfonation (Seth et al, 2000; Gray et al, 2009).

Additionally, it has been shown that CYP1A activity is inhibited by alkylphenols in several fish species, which may have consequences in clearance of xenobiotics, resulting in the accumulation of harmful compounds. Besides its detoxifying function, CYP1A also mediate the metabolism of xenobiotics, a process that can also lead to the formation of reactive oxygen species (ROS) (Sturve et al, 2006).

Finally, other fact to take in consideration is that the hydrophobic alkyl residue can induce alterations in cell membranes, by increasing membrane permeability and causing cell leakage and loss of ions (Bhattacharya et al, 2008). Watson et al (2010)

refer that alkylphenols was quite potent in several performed assays for nongenomic responses, including PRL release, cell proliferation, calcium (Caþþ) influx, and in the activation of mitogen-activated protein kinases.

## PRE-INITIAL FOCI (PRE-FCA)

During the observations we verified that FCA seemed to have an early stage of poorer definition, a feature that, to best our knowledge, was never emphasized before and/or distinctly described in the literature — so, and at least provisionally, we called those stages as Pre-FCA. This naming presumes that, if evolving, the change will turn into a well-differentiated type of FCA. After careful considering all the variable aspects seen in our material, we advanced the following criteria for the classification of this type of finding:

(1) Lesion composed by less than 12 cells (in a 2D-perspective, i.e., when sectioned);

(2) Presence of a mixture of normal and altered hepatocytes, making the lesion heterogeneous (making it difficult the limitation of the lesion, in more than 50% of its perceived boundary);

(3) Lesion already presenting a tinctorial change, however without a trabecular aspect or any increase of the vascularization.

It must be stressed that Pre-FCA were not easy to detect a low magnification when the normal parenchyma was normally basophilic, being the digital microscopy a very useful tool in these cases. Besides the difficulty in diagnosing on those occasions, we may reason that Pre-FCA can be a peripheral part of a differentiated FCA; derived from a tangential section of the FCA. To discern this, the second and third criteria above were essential. In cases where the hepatocellular parenchyma presented a high level of heterogeneity — i.e., with hepatocytes displaying a more vacuolated aspect in some areas in parallel with more basophilic tinge in other zones — and a higher number of sectioned FCA, the Pre-FCA identification was almost impracticable; very common situation in E2 50 µg/L group.

In the end, the differential diagnostic between Pre-FCA and well-established FCA is possible, and in our opinion, this distinction can have a major significance. For example, the detection of this early stage can point to the possibility of a reversible stage. Under this presumption, if the promoting stimulus stopped the Pre-FCA could disappear and not evolve to FCA, and further to hepatocellular adenomas (HCA) and ultimately to HCC. Myers et al (1987) even suggested that FCA can be back into normal parenchyma if the stimulating contaminant is removed, an assumption mentioned

recently by Oliva et al. (2013), whom pointed it like a possible complicating factor in the interpretation of data for lesions. Thoolen et al. (2012) also referred the same and added that different types of FCA had different potentials for developing into neoplasms. Anyway, both the nature and the reversibility potential of Pre-FCA and FCA deserve detailed experimental modeling, namely because they early signal significant risks, in an ecotoxicological context and individually.

## DIAGNOSIS AND IMAGING ANALYSIS: DIFFICULTIES AND FINDINGS

One true challenge for diagnosis of the pre-neoplasms was the great heterogeneity of the parenchyma in certain animals and/or the fact that sometimes all the liver seemed like an immense focus; two situations that turned the limitation of the focal lesions very difficult. We hypothesized that the described scenario should be viewed as "normal", considering that the liver (at least of exposed animals) was being chronically challenged to detoxify the xenobiotics reaching the blood stream and ultimately the liver. Such kind of histological heterogeneity of the all parenchyma (and eventually an adaptive change) was not particularly stressed in previous carcinogenesis assays, but, in theory, it should have metabolic implications. This issue will be discussed further below, when dealing with the comparison of the seemingly normal parenchyma that we analyzed in every group.

Other difficulty concerned the foci at the margins of the sections, because they can be mistaken with histological artifacts, like tissue compression or staining accumulation, or with parenchymatous peripheral alteration. These aspects had not been addressed in the literature, but they should be reported so that common diagnosis criteria are established.

The classification between basophilic, eosinophilic and amphophilic was not a linear task as it may seem — namely in view of the reasonably clear published criteria — because FCA can be heterogeneous, comparing intra and inter sections within slides, in which concerns staining or aspect, both influenced by the parenchyma type itself. For instance, in a vacuolated parenchyma, the detection was faster and precise, since FCA, rich in determined cytoplasmic constituents; easily stand out by the staining. Boorman et al. (1997) previous stated that it is complex to diagnose the foci just based on staining, as the sex of the specimen, section thickness, or the staining qualities of a particular lesion can influence the aspect of a particular FCA to the observer; impacting on the diagnosis.

When diagnosing the lesions we observed that FCA were preferentially located at perivascular zones (50% of cases), a feature that was more evident in the more ex-

tensive FCA. This points to the question: besides the increased capillary dilatation, and thus of blood supply that is always associated with increased the trabecular aspect, is there also preneoplastic neoangiogenesis (with formation of new blood vessels) that significantly contribute to the foci development and the later neoplastic transformation? Alternatively, is there a trend for the FCA to develop near larger blood vessels because initiation and/or promotion factors are more available on those areas? These facts are worth investigating, and to our knowledge, the FCA spatial trends of localization have not been addressed yet.

Another interest fact herein was the frequent fragility of the sections in the FCA spots, suggesting the possibility of intercellular adhesion be more fragile there. Another option is that it is simply an artifact, but this seems unlikely because of the frequency of the event.

## THE NORMAL PARENCHYMA

Based on the clear difference observed between the cell and nuclear volume of hepatocytes from the blank control group and from all the four groups with lesions, we can reason that the presence of lesions (i.e., of an undergoing pre-neoplastic process) leads to the creation of a microenvironment that results in significant morphological alterations in the hepatocytes; which may be viewed as a proxy of functional differences too. The morphological hypertrophy of the hepatocytes and their nuclei, regardless of estrogenic stimulation, can be viewed as a physiological adaption in response to some greater functional need or to other stresses (e.g., Wei et al., 2012). By other hand, that kind of cell hypertrophy could result from some kind of signaling coming from the FCA. Hepatocyte hypertrophy is a well-known first response to the removal of liver mass, and is controlled by several local (complex and ever increasing) signal network (Miyaoka and Miyajima, 2013) — eventually, as the FCA emerge and progress they could signal a “loss of healthy liver”, inducing bigger cells in the normal parenchyma. Facing the background, this interesting hypothesis does not seem too speculative to us and could be investigated futurely, in fish and also in a better known rodent model.

Looking to the $CV_n$ ($\nu$) we verified that the nuclear heterogeneity was elevated in all the groups with lesions (being statistically different from the blank control), possibly pointing to an increase in the degree of morphological diversity/dysplasia. This parameter was computed only for the nuclear volume since this factor has been used as a proxy of cell heterogeneity or even dysplasia, which is logical given the fact that the nuclei is the metabolic center of the cell.

Our results assume relevance since we verified that changes occur in the normal hepatocyte phenotype, additionally to those already observed at a molecular level, as described for instance by Mirbahai et al. (2011), who stated that global methylation was statistically significantly reduced in hepatocellular adenoma and in non-cancerous surrounding tissues, when compared with livers from non-cancer bearing dab (*Limanda limanda*, a flatfish). In the same vein, Roskams et al. (2000), in an attempt to discover if the hepatocellular cancer influence/induced suicide in peritumoral cells, also verified a high co-expression of Fas (factor receptor apoptosis mediator) and Fas-L (Fas ligand) in hepatocytes immediately adjacent to HCCs. This again supports the notion that neoplastic (and eventually the pre-neoplastic lesions) lesions do influence the normal hepatocytes, which may underlie the morphofunctional changes that are found in our study and other. Thereby, these morphofunctional alterations identified in the apparently normal parenchyma can help researchers to recognize, at early stages, that lesions are emerging, calling for the development of integrated biomarkers to detect them.

## ALTERED PARENCHYMA: VOLUME PARAMETERS OF bFCA AND aFCA

The results from the comparison between the four fish groups having FCA, regarding the several stereological parameters from bFCA and aFCA, meet with previous outcomes, which highlight the powerful estrogenic potential of E2 at 50 µg/L, and support the hypothesis of a protective effect of E2 at 5 µg/L; which continuous to trigger the lowest values. Somewhat surprisingly, the bFCA and aFCA in the Alkylphenols group and even from Vehicle Control overlapped the E2 50 µg/L group regarding $\bar{v}_n$ (hepatocyte). This means that the hypertrophy of the nucleus is not strictly proportionally accompanied by all the cell volume, resulting in an elevated ratio nucleus/cell. Besides that, with these results, it became more probable that alkylphenols may trigger different cellular mechanisms when compared with E2, despite their action being mostly associated with similar (usually milder) effects of estrogens. It is also interesting to note that in the analysis of the Table 7 and 8, which included every type of FCA, the groups did not seem to differ from each other, only from blank control, and now we can clearly verify the differences, fundamentally between the E2 5 µg/L and E2 50 µg/L and the remaining groups; whereas fish from Alkylphenols and Vehicle Control groups still present similar outcomes. Thus, the global analysis, involving the three types of FCA, differs from the individual, which points to the importance of sub-groups identification.

Observing the results of the $V_V$ (sinusoid) within the FCA, the groups are not different from each other, indicating the possibility of an optimal level of vascularization for the development of FCA. Concerning the $V_V$ (nucleus) within the FCA, the estrogen-

ic impacts seen in the E2 5 µg/L and E2 50 µg/L groups surpassed those of the Vehicle Control, in both bFCA and aFCA, according with the trend for increased of the ratio nucleus/cell, especially at the highest E2 concentration, and in line with the well-known fact that such an increased ratio and amplified presence of nuclei are associated with pre-neoplastic dysplasia. In short, the data support a higher impact on hepatocytes as estrogenic dose increases.

## HISTOCHEMISTRY

The Perls' staining is commonly used in liver to examine the presence of ferric iron (Fricke et al, 2012; Dabrowska et al 2012; Myers et al, 2003). The results herein were not the expected, since we did not verify the resistance of iron uptake and deposition in FCA as suggest by Feist (2004). We can argue that this difference may be connected with factors, such as type of initiator, the estrogenic stimuli, the species, among other, thus warning against generalizations when the knowledge of fish liver carcinogenesis is still meager.

Sirius red staining has also been used in liver lesions to detect collagen deposits, particularly pericellular material surrounding degenerative hepatocytes (He et al, 2013; Hobbie et al, 2012). In this case, we resource to Sirius red to visualize a possible thickness of blood vessels inside the FCA, by staining the fibrillar collagen. Comparing the vessels in the normal parenchyma with those in the FCA, we did not observe significant differences, however, when we compared both situations with the scenario in an adenoma the scenario changed, and we detected a slight increase in thickness of the blood vessels. In the Japanese medaka (*Oryzias latipes*), a stronger Sirius red staining in animals exposed an initiator agent was interpreted as an increased collagenous matrix deposition due to the restructuring of the hepatic architecture after the injury (Hobbie et al 2012). In our case, as the lesions, and particularly, the hepatocellular adenoma evolves, the architecture is expected to change along with hypervascularization.

On the other hand, glycogen-storing is likewise a relevant aspect analyzed in hepatic tissue, given the several associated diseases (Özen, 2007), and even in hepatocarcinogenesis (Banasch et al, 2003). As mentioned before, the type of FCA with increased glycogen storage is the clear cell one, so PAS staining can be used to diagnosis this foci, remembering, however, that glycogen may be partially lost because of the histological process. Based on the possible evolution of the FCA, explained also above, we expected not to detect any trace of glycogen in eosinophilic foci and a possible slight occurrence in basophilic foci. Sato et al (2013), when observing spontane-

ous amphophilic foci in a young rat, stated that glycogen content of the hepatocytes within the lesion was relatively less comparing with the normal area, whereas some hepatocytes had abundant glycogen. This fact was also previously verified by Mayer (2003), in a study based on the induction hepatic pre- and neoplasia in the rat with dehydroepiandrosterone, where she describes amphophilic foci as a lesion with a very low glycogen content and low activity of key enzymes of glucose metabolism, high activity of mitochondrial and peroxisomal enzymes, as well, lysosomal acid phosphatase. Our results were different, more complex, since we saw clear heterogeneity among the amphophilic FCA, visualizing in some cases more glycogen within the FCA than in the surrounding parenchyma. By the contrary, basophilic and eosinophilic FCA, and even the adenoma, presented less glycogenic content comparing with the parenchyma. Here we were expecting to verify an aspect to support the evolution line, but we found a heterogeneous middle stage (amphophilic FCA) and equal scenario in opposite phases (basophilic and eosinophilic FCA). However, based on the majority of the studied cases, it seems to be a deficiency in glycogenic component in FCA comparing with the surrounding parenchyma, which is in accordance with previous studies. Anyway, our data reveals that FCA of the same type may reveal dissimilar histochemical profiles.

## IMMUNOHISTOCHEMISTRY

E-cadherin, as a key cell adhesion protein, is implicated in a variety of morphogenetic events, including cellular growth and transformation, cell migration, separation and formation of boundaries between cell layers and differentiation of each cell layer into functionally distinct structures. Since changes in adhesion complexes may lead to alterations of cell polarity, proliferation, mobility, and differentiation, E-cadherin plays a critical role as invasiveness suppressor in carcinomas (Wei et al, 2002; Calvisi et al, 2004).

In a diversity of cancers, reduced expression of E-cadherin has been correlated with disruption of cell-cell contacts, epithelial-mesenchymal transition, invasiveness, and metastatic potential (Wei et al 2002), being a frequent finding in liver cancer (Prange et al, 2003) and its down-regulation closely related to the progression of HCC (Matsumura et al, 2001; Kwon et al, 2005). Our results support these previous studies, since we observed a negative pattern in E-cadherin immunoreactivity within the FCA, differing from the surrounding parenchyma. It is a very interest fact, since we can verify the loss of this protein in a so early phase of the lesion, a feature that probably has a critical role in FCA progression. We wondered whether or not there are differences of E-cadherin staining between different types of FCA, but in this approach we did not

have the time to investigate this issue further. Yet, it is worth checking it in the future facing the differential diagnose potential.

Cytochrome P450 (CYP) plays a crucial role in oxidative metabolism of lipophilic drugs and xenobiotics (possible carcinogens). During hepatocarcinogenesis, the expression of drug metabolism enzymes can be altered, being CYP induction influenced by a number of exogenous and endogenous factors (Liu et al, 2005). Also, it already described that modification of CYP activities in rats can be a determinant factor for the resistance *vs* susceptibility to xenobiotics in the early hepatocarcinogenesis stages (Maliakal et al, 2002). The CYP decreased expression in preneoplastic lesions could alter the synthesis and/or inactivate endogenous and/or exogenous substance, which act as tumor promoters (Liu, 2005). On the other hand, Jaeschke et al (2001) concluded that induction of CYP isoenzymes can promote oxidant stress and cell injury.

CYP1A, one of the main cytochrome P450 enzymes, can function as marker to characterize the carcinogenic potential of xenobiotics (Liu, 2005). Androutsopoulos (2009) stated that CYP1A1 can play an important role in the detoxification of environmental carcinogens, thus contributing to prevent cancer, or act in procarcinogen activation and lead to cancer progression. So, several P450 substrates are carcinogenic, while other substrates are anti-cancer drugs, resulting in its different potentially important roles in tumor biology and progression (Murray, 2000). We observed herein heterogeneity between aFCA and eFCA, with distinct patterns of CYP1A immunoreactivity, ranging from very weak to strong signals. Only in bFCA, we verified a more linear pattern, with the consistent weaker intensity. Once again, we were not able to find a feature to clarify the “evolution line” of FCA, observing a heterogeneous middle stage (aFCA), and a similar scenario in “opposite” foci phases (i.e., bFCA and eFCA). These results strongly point for the possible existence of different phenotypes within same type of FCA, which is poorly/non-studied issue, but that could help explain, for instance the fate of individual FCA irrespective of the type. The data point also for a more complex scenario that usually referred in the literature, that often presents the FCA as a unique homogenous entity, when, in the light of the fact that a tumor (inc. hepatocellular carcinomas) may be monoclonal or polyclonal, homogenous or heterogeneous (Kawai et al, 1995; Marusyk et al, 2010), it makes sense to reason that a type of FCA may indeed correspond to multiple morphofunctional entities according to the underlying genetic/epigenetic events.

Regarding the immunohistochemistry, we were only able to perform a first approach that nevertheless proved to be successful, with promising discriminative results, suggesting that in the future this subject should be exhaustively studied with a ample

panel and number of cases. Indeed, it is our intention to advance in this vein in the next future.

# CONCLUSION AND PERSPECTIVES

## CONCLUSION AND PERSPECTIVES

In the end of the work we were able to respond to the proposed objectives described in the Introduction, opening good perspectives for the continuation of the study.

The virtual slide and the image analysis measurements obtained with the basic slide scanner software proved to be beneficial to the efficiency on this type of screening diagnosis and quantification, and we recommend it for further studies of the same sort.

The protocol for inducing the emergence of a carcinogenesis process based on a single initial bath of eye-egg embryos in a water solution of MNNG was successful. However, the time from exposure to sampling allowed the emergence of preneoplastic lesions only, mostly FCA and a couple of hepatocellular adenomas. For ending up with carcinomas, either the dose of MNNG and/or the time up to sampling should increase.

It was clear that estrogenic stimulation can act as a promoter, being E2 at high dose more effective when compared with the alkylphenols mixture. Indeed, the group E2 50 µg/L stood out with greater frequencies (100% in FCA prevalence), a tendency also noted in the volume parameters. We were not expecting that the results group E2 5 µg/L (namely, the lowest FCA prevalence) pointed for potential paradoxical effects of estrogens — protector and promoter. Thus, it became difficult to predict the outcomes since they apparently depend on the doses and even when chemicals seem to assume a low dangerousness, we cannot rule out the possibility of risks at long time, stressing the importance of a continuous monitoring when exposure exists. Notwithstanding the alkylphenols mixture lowest estrogenic potential then E2 at 5 µg/L group, the fact is that it may trigger more damaging effects (at least in view of the parameters considered in this study), eventually by combining a xenoestrogenic action with other different cellular mechanisms/pathways.

The classification of FCA was not so linear as we could thought from the literature, but despite some uncertainties during certain diagnoses, they were not major obstacles. During the observation of the slides, we were able to establish differential criteria for what seem to be FCA in early stages and poorer definition, which we called Pre-FCA. It was also interesting to verify that, when parameters were compared in a global analysis (summing all three types of FCA) and then in type-by-type analysis, the results provided new insights, proving the importance of sub-groups identification.

Our data revealed that the same type of tinctorial FCA may express dissimilar histochemical and immunohistological profiles, leading to a complex scenario and presenting the FCA as multiple morphofunctional entities. This possible existence of different phenotypes did not help us to clarify the “evolution line” of FCA, adding to the fact that some of our results did not agree with the most usual idealized path, where

bFCA is viewed as the preneoplastic stage that precedes the HCC. However, in this approach, we did not have the time to investigate this issue further in terms of special histo/immuno-tagging techniques, namely in view of its differential diagnose potential.

On the other hand, our results pointed to previously unidentified changes that occur in the apparently normal parenchyma, in presence of FCA, which not only suggest some sort of influence of the carcinogenesis process in the all liver, even if not clearly lesioned, but open the door for using such information as part of a biomarker panel that could to recognize, in an early stage, that neoplastic lesions are emerging.

Future studies of these presumptive preneoplastic lesions, especially at a molecular level, should help to characterize with more detail each type of FCA, clarifying its evolution line, defining their role in progression to malignancy and even testing the existence of a reversible stage. As reasoned, this can lead to biomarkers for early diagnosis, prognosis and interventions, both in terms of environmental health and eventually even in possible preventive/treatment approaches. The findings of this work, complemented with further investigation, should also provide a basis for understanding liver responses after exposure to estrogens post-initiation, which can predict risks and dangerous outcomes, not only for fishes exposed to pollutants but even up to Humans.

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